Replicative Bypass of a DNA-peptide Cross-link and Oxidative Single and Tandem DNA lesions in Human cells

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University of Connecticut, 2020

DNA harbors the genetic information in all living organisms and plays a pivotal role in passing the genetic information to the next generation. All living cells are exposed to numerous exogenous and endogenous agents that damage the DNA to form different lesions or adducts in DNA. Fortuitously, diverse DNA repair mechanisms exist in the living cells, which repair these DNA adducts before the replication apparatus of the cell copies the DNA. However, frequently extensive DNA damage or slow repair may allow replication bypass of a DNA lesion. As a result, cells adapt a DNA damage tolerance mechanism, termed as translesion synthesis (TLS) to bypass the lesions in DNA. Often, the TLS polymerases are of low fidelity and result in the misincorporation of the nucleobases generating mutations. If these mutations persist in the DNA, it may lead to alteration of genetic code and protein expression, thereby resulting in various diseases including cancer.

In this thesis, I investigated the mutagenicity and genotoxicity of three different adducts including a bulky DNA-peptide cross-link, a tandem lesion containing 2-deoxyribonolactone with thymine glycol and oxidative stress induced formamidopyrimidine dG (Fapy.dG) in human embryonic kidney (HEK293T) cells. My goal is to investigate the role of each of the TLS polymerases (pol **η**, pol **κ**, pol **ι**, pol **ζ**), by replicating these lesions in selective polymerase-deficient human cell lines and determine the types and frequencies of mutations. From these analyses, it was discovered

that these adducts are bypassed by TLS mechanism in both error-prone and error-free manner by the action of the specific TLS polymerases. From these studies, it was evident that the replicative bypass depends of the structure, DNA sequence context, and the orientation of the lesion in DNA. This research provides an insight to better understand the mutagenicity and toxicity of these lesions in human cells, which should benefit gene therapy applications in the future.

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Dedicated to

My mom, dad and sister, Pravallika Snehitha

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CHAPTER – 1

INTRODUCTION

Cancer is a multi-step process, triggered by the abnormal proliferation of cells. It is also the second leading cause of death worldwide. Most common types reported are lung, breast, colorectal, and prostate cancers (1). According to international agency for research on cancer (IARC) (2), 9.6 million deaths were reported in 2018, with lung cancer being the leading cause in both men and women. As per IARC statistics (2), 11.6 % new cases reported were of lung and breast cancers, followed by 10.2 % of colorectal, 7.1 % of prostrate, 5.7 % of stomach cancers. Abnormal cellular proliferations are provoked by several factors such as, inheritance of genetic disorders, abnormal expression of transcription factors, formation of successive mutations in the genes including the tumor suppressor gene, *p53*. Different types of cancers are developed from several gene mutations, which are formed primarily from the replication of DNA damage (1,3). These mutations alter the gene expression in the cells and promote carcinogenesis. Carcinogenesis is a multi-step process which include initiation, promotion, and malignant progression (4). The initiating agent becomes a carcinogen when treated at higher or repeated dosages, initiate malignant progression. The promoting agent is a non-genotoxic agent but act synergistically with the initiator to promote the progression of cancer (4). Several carcinogenic agents such as chemical compounds from cigarette smoke, diesel exhaust, charred meat, physical agents such as radiation, microbial agents (bacteria, viruses) may result in the dysfunction and alteration of gene expression in the cells, lead to cancer and various other diseases.

Other implications due to accumulation of gene mutations are premature aging or death of the cells. The somatic mutation theory of cancer proposes that (5, 6) the accumulation of non-lethal

mutations (driver mutations), when associated with mutant cell proliferation causes cancer. Effects of this association results in chromosomal aberrations, developmental disorders, rare genetic diseases etc. Fanconi anemia (FA) is a rare genetic disorder that occurs due to the mutations caused in 21genes, which have a role in repair of the inter and intra-strand cross-links (ICLs) in DNA (7, 8). The effects of FA include chromosomal instability and bone marrow failure. Accumulation of somatic mutations in the cells may also lead to developmental disorders and cancer such as melanoma. Epigenetic modifications such as DNA methylation, covalent histone modifications, non-coding RNAs such as miRNAs in the cells are essential for mammalian gene expression (9). However, any disruption will lead to the genetic alteration and cancer. For instance, hypomethylation and hypermethylation of DNA will alter the gene expression which are responsible for cell cycle control, repair and apoptosis (9). Hence it is extremely important to study the etiology of gene mutations caused by various DNA damages in order to explore the treatment methods to cure these genetic diseases.

LITERATURE SURVEY

1.1 DNA DAMAGE:

DNA contains the genetic information, vital to the survival and propagation of all living organisms. Every human cell is exposed to various endogenous and exogenous agents that damage the DNA; it is estimated that about 70000 lesions form per day (3,10). These DNA damages may occur spontaneously or due to the prolong exposure to various chemical and environmental agents. It is estimated the about 10^5 spontaneous DNA lesions are formed per day (10). If left unrepaired, the lesions undergo low fidelity replication which generate gene mutations, thereby leads to tumor progression (4).

Endogenous DNA damage in the cells include reactive metabolites like reactive oxygen species (ROS), reactive nitrogen species (RNS) produced as a result of oxidative stress. ROS includes hydroxyl radical ($OH^{\bullet}$), superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). The highly reactive hydroxyl radicals ($OH^{\bullet}$) form tandem lesions, formamidopyrimidines, strand breaks, and various purine/pyrimidine modifications (11-13). Other endogenous processes such as deamination reactions induce conversion of cytosine to uracil and SAM-induced methylation forms 3-methyladenine (3meA) and 7-methylguanaine (7meG) (3). Some of these processes may occur spontaneously as in the case of depurination/depyrimidination resulting in abasic sites (12).

Exogenous DNA damage include exposure of DNA to many environmental agents. These environmental sources can be either physical or chemical agents (3). Physical agents include UV and ionizing radiation (IR). UV induces the formation of cyclobutane pyrimidine dimers (CPDs), 6-4 photoproducts (PPs) (11). X- rays induce oxidation of the nucleobases, formation of single strand breaks (SSBs) and double strand breaks (DSBs) (14). Chemical agents induce the formation

of various bulky and non-bulky DNA adducts in the cells, which may disturb the architecture of the DNA helix. These alterations may influence the replication process to generate mutations. Some of these are mentioned here and studied in this thesis. Simple alkylating agents such as methylmethanesulfonate (MMS) and temozolomide form *N*3-methyladenine, anti-cancer drugs such as mitomycin C (MMC), cisplatin, cyclophosphamide and nitrogen mustard induce inter-strand cross-links (ICLs) (3). Topoisomerase inhibitors such as camptothecin (CPT), etopsin, formaldehyde induce SSBs or DSBs and DNA protein cross-links (DPCs) (3,15). The chemical compounds in cigarette smoke (nitrosamines, NNK, NNN) (16), diesel exhaust (3-nitrobenzanthrone, 1-Nitropyrene) (17, 18), coal tar (polycyclic aromatic hydrocarbons (PAHs)) (19), dyes, industrial chemicals in the preparation of PVC (vinyl chloride, 1, 3-butadiene) (20), aflatoxins (food contaminants) (21), charred meat (aromatic amine IQ) (22) metabolize in the cells to produce bulky DNA adducts and promote tumorigenesis.

1.1.1 DNA protein cross-links (DPC):

DNA protein cross-links are a class of bulky DNA adducts formed when the nucleobase of DNA covalently bonds with an amino acid moiety of a protein or peptide (23, 24). Due to their bulky size and helix-distorting property, DPCs disrupt the replication and transcription process which may be fatal to the cells. DPCs are induced by various sources categorized as environmental, therapeutic, and endogenous (24). DPCs formed by various agents are chemically distinct and also depend on the cell cycle phase, cellular metabolism, and temperature. Environmental sources include UV light, IR (ROS, RNS), metal ions like chromium, nickel, carcinogens like diepoxy butane, acrolein, crotonaldehyde, 1,3-butadiene (25-29). Therapeutic sources include the anti-cancer drugs like cisplatin and nitrogen mustard (23, 30-33).

Endogenous sources may also result in either non-enzymatic or enzymatic DPC formation (24). Non-enzymatic pathways include a majority of reactive aldehydes such as formaldehyde and acetaldehyde, which form DPC. DPCs may also form as intermediates in the processes such as amino acid metabolism, oxidative demethylation (25, 34). Most extensively studied DPCs are formaldehyde-induced adducts (FA-DPC). It is reported that some FA-DPCs are reversible and may involve a broad variety of proteins (35, 36). These FA-DPCs are previously studied *in vitro (37)*, and also in *S. cerevisiae (38)* and human cells (39). Other non-enzymatic sources include the formation of DPC through abasic sites. These abasic sites contain reactive aldehyde groups, which when react with the proteins and smaller peptides, form DPC and DNA peptide cross-links (DpCs) (27, 37, 40).

Enzymatic sources include topoisomerases, DNA glycosylases, MGMT, PARP-1 enzymes, which form transient complexes with the DNA as part of its mechanism but in certain conditions they can get trapped onto the DNA to form DPCs (41-43). DNA glycosylases and PARP-1 enzymes are involved in the formation of abasic sites as part of base excision repair (BER) mechanism, but these sites are prone to covalent cross-linking with the enzymes itself to form DPC especially by short-patch BER (44, 45). Studies have been conducted both *in vitro (15)* and *in vivo* (39) to explore the mutagenicity and cytotoxicity of DPCs as well as DpCs, by generating synthetic site-specific DNA cross-link adducts. DPCs can also be repaired by nucleotide excision repair (NER), due to the bulkiness of the lesion (46).

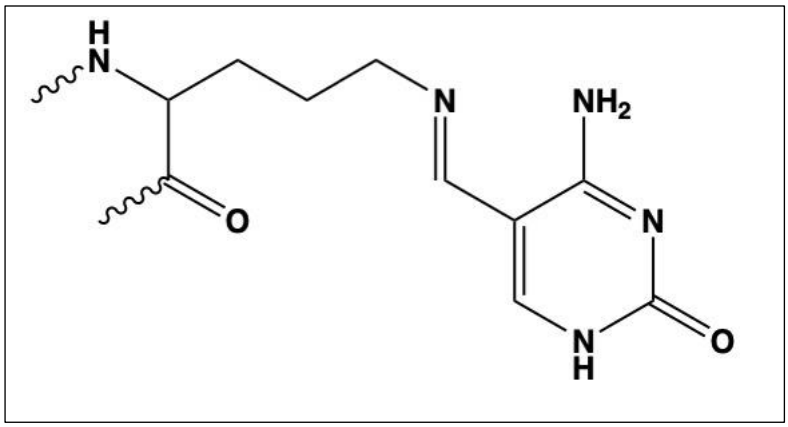


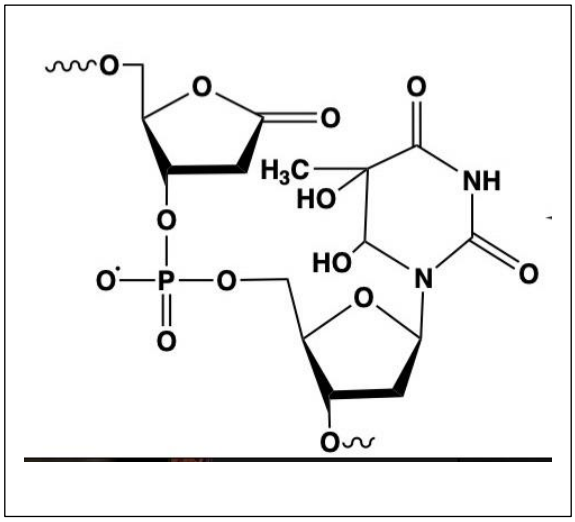
FIGURE 1.1: DNA peptide cross-link with lysine of peptide covalently linked to cytosine of DNA

1.1.2 Abasic sites:

Apurinic/apyrimidinic (AP) sites in the DNA are referred to as abasic sites. AP sites can be generated due to spontaneous loss of a base, by alkylating agents or by the DNA *N*-glycosylases, which remove the modified base at the damage site, as a step in BER process. The rate of depurination is higher than depyrimidination of DNA in the cells (47). AP sites are formed by spontaneous hydrolysis of the glycosidic bond between the deoxyribose sugar and the nucleobase along with the disruption of the phosphodiester bond with the neighboring nucleobases in the DNA (48). It is reported that about 10000-50000 abasic sites are produced in the mammalian cells per day under aerobic conditions (49, 50). Replication of these abasic sites may result in ICLs, DSBs and histone modification (51-53). These abasic sites are highly electrophilic and chemically unstable. Oxidized AP sites react with the DNA repair enzymes and inhibit their function. They are also reported as highly mutagenic and generate single base-pair substitutions in *E.coli (54).*

Oxidized abasic sites are produced by ROS, when exposed to higher levels of IR or prone to oxidative stress (55). These sites also generate DPCs by covalently cross-linking to the different

enzymes and block the replication and transcription process. One such important oxidative abasic lesion is the formation of 2-deoxyribonolactone (L). Formation of L also occurs when DNA is exposed to Cu $(OP)_2$ and antitumor agents such as Neocarzinostatin chromophore (NCS) (56, 57). The absence of the nucleobase exposes the C1'-H bond of the deoxyribose backbone which is prone to the oxidation and under aerobic conditions, produce L (58, 59). Abasic sites are repaired mainly by BER and to some extent by NER when the former repair process is disrupted (60, 61). L is generated as an isolated lesion as well as a part of tandem lesion by the attack at C1'-H bond as described above (62, 63).

A. 

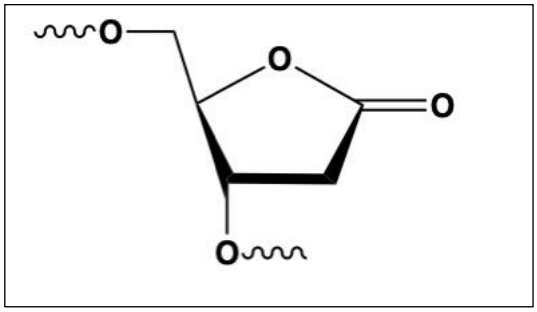
B. 

FIGURE 1.2: (A) 2-deoxyribonolactone as tandem lesion with thymine glycol (LTg) and (B) 2-deoxyribonolactone as isolated lesion (L).

1.1.3 ROS:

Reactive oxygen species (ROS), its corresponding radicals and non-radicals are generated as byproducts in the aerobic metabolism (64, 65). Low levels of ROS are beneficial to the living organisms as they act as signaling molecules in regulating the biological and physiological processes like cell cycle progression. High levels of ROS will react with biomolecules such as lipids, proteins, and DNA, causing damage. ROS includes superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and highly reactive hydroxyl ($OH^{\bullet}$) radicals (11, 12, 66, 67). Different ROS react with the nucleobases (dA, dT, dG, dC) to form various DNA lesions (68). Of the four bases, ROS reacts with dA and dG to a greater extent (67). The oxidatively damaged DNA is efficiently repaired by BER (61, 69).

Free radicals like $OH^{\bullet}$ react with nucleobases by addition followed by abstraction pathway. The radicals attack the C5 and C6 position of pyrimidines (dT, dC) and add OH groups, thus generate free-radicals and further abstracts the hydrogen atom (12, 70, 71). These C5 and C6 free-radical containing intermediates under aerobic conditions will produce various lesions. The most notable lesions are thymine glycol, 5- formyluracil, 5- hydroxycytosine, 5- hydroxyuracil. For purines (dA, dG), the $OH^{\bullet}$ radicals attack C4, C5 and C6 positions to generate free-radicals (70). Under aerobic conditions, these radicals form various lesions such as 8-oxopurines (8-oxodG, 8-oxodA) and formamidopyrimidines (12, 67, 70). Extensive studies were performed to understand the replicative and repair processes of 8-oxopurines in *E.coli (72)* and human cells (73-75). The formamidopyrimidines are 2, 6-diamino-4-hydroxy-5-formamidopyrimidine (FapydG) and 4,6-diamino-5-formamidopyrimidine (FapydA), of which the earlier lesion is more notable studied (76, 77). These $OH^{\bullet}$ radicals also form the tandem lesions, in the presence of O_2 and the targeted base is 5' to the pyrimidine (78, 79). For instance, attack of thymine with $OH^{\bullet}$ under aerobic

conditions will result in the formation of hydroperoxyl radical; this in turn attacks either dA to form the tandem lesion, 5'-2-deoxyribonolactone -thymine glycol -3' (5'-LTg-3') (62, 63).

In this thesis, the implications of some of the DNA damages such as tandem lesion LTg, isolated lesions L and Tg, bulky DpC, ROS-induced FapydG was studied in human cells and are reported in the subsequent chapters.

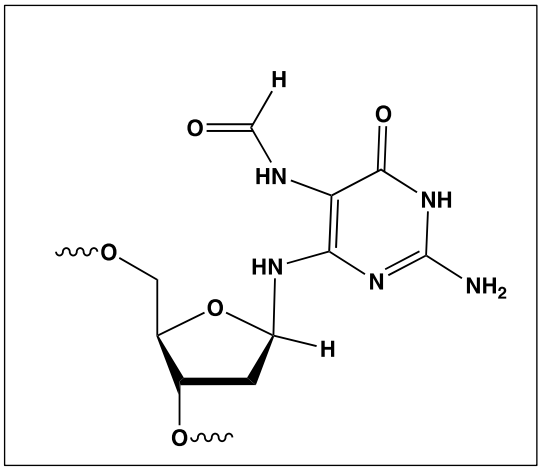


FIGURE 1.3: Structure of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapydG).

1.2 DNA REPAIR:

DNA damage occurs in all the living organisms and fortunately, to tackle these disruptions, DNA damage responses are activated in the cells. If these damages cannot be repaired the DNA may undergo replication by error-prone translesion synthesis (TLS) generating high frequency of mutations. Alternatively, if this damage blocks the cellular processes, the cell is subjected to apoptosis (61, 80-82).

DNA damage responses in the cells include the following,

- Removal of DNA damage by repair mechanisms
- Activation of DNA checkpoint to arrest the cell cycle progression
- Altering the transcription profile in order to modify the gene expression.
- Programmed cell death (apoptosis)

Different types of damages are recognized by various factors, which trigger the corresponding DNA repair mechanisms. These are categorized into four types:

1.2.1 Direct repair

1.2.2 Base excision repair

1.2.3 Nucleotide excision repair

1.2.4 Mismatch repair

1.2.5 Homologous recombination and Non-homologous end joining

1.2.1 Direct Repair:

Two mechanisms of direct repair include the enzymes photolyase and methylguanine DNA methyltransferase (MGMT) acting at the DNA damage site (61, 80). Photolyase repairs UV-induced lesions cyclo-pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4 PPs) by direct reversal (83). This repair mechanism does not occur in mammalian cells, in which instead the cells adapt NER to repair these lesions. MGMT removes the alkyl groups from the pyrimidines to convert into normal bases. This enzyme converts O^6-methyl guanine into guanine by eliminating O^6-methyl group (83).

1.2.2 Base Excision Repair (BER):

Spontaneous DNA damages which significantly doesn't distort the double helix, are repaired by BER pathway (80). For example, the damages caused by deamination, alkylation and oxidation by various agents as discussed above (69). BER occurs both in nucleus and mitochondria of a living cell and it prevents the cells from aging, cancer, and neurodegeneration (69). BER pathways begin with the recognition of the damaged site by DNA glycosylases, which are lesion specific and removes the damaged base to form an abasic (AP) site (49). Based on the type of glycosylases recruited in the base excision step, the repair proceeds in two ways, one is short-patch BER (1-base removal) and another is long-patch BER (2-10 bases removal) (60, 69, 84).

A. *Short-patch BER (69)**:***

In the first step, the AP site is created by action of *N*-glycosylases to remove the damaged base, followed by AP lyase, which cleaves the phosphodiester bond at 3' end of the AP site. Then, AP endonuclease1 (APE1) cleaves the 5' end of the phosphodiester bond to create a gap. Next, pol **β**

is recruited to insert a single nucleotide complementary to the template in the gap. Next, the gap is sealed by ligation using ligase- III/ XRCC1 complex.

B. Long-patch BER (69,85):

In this process, the AP site is formed by hydrolytic glycosylases followed by the cleavage of the phosphodiester bond at 5' end of the AP site by APE1 enzyme. Then proliferating cell nuclear antigen replication factor C (PCNA-RFC) complex recruit high fidelity replicative polymerases **δ**/**ε** to carry out the insertion of new bases by displacing the existing ones. This 5'- displaced nucleotide stretch is cleaved by flap endonuclease-1 (FEN1) and the gap is sealed by ligation using ligase-I enzyme.

1.2.3 Nucleotide Excision Repair (NER):

Broad range of bulky or distorting adducts are repaired by NER such as bulky DPCs, UV-induced lesions (CPDs, 6-4 PPs), adducts from polycyclic aromatic hydrocarbons (PAHs), aromatic amines, oxidative induced cyclopurines, adducts formed from chemotherapeutic drugs like cisplatin, ICLs and so on (12, 46, 86). The lesions which are bulky and thermodynamically destabilize the DNA helix make very good substrates for NER. There are categorized into two types based on the platform where it occurs, namely transcription-coupled NER (TC-NER), which repairs the genes in the transcribed strand and global genome NER (GG-NER) (87), which repairs the genes at any stage of the cell cycle and location in the genome. Deficiencies in the NER repair proteins will lead to skin cancer related genetic disorders such as xeroderma pigmentosum (XP), Cockayne's syndrome, trichothiodystrophy. In this repair process (88), the lesion containing strand is recognized by the protein complex XPC/ RAD23B, by binding to the non-damaged strand at the

site opposite to the lesion. The factor TFIIH interacts with the XPC-RAD23B and unwinds the DNA by its subunit XPB. The unwinding proceeds until its other subunit XPD encounters the lesion, then recruits XPA, RPA, XPG to form pre-incision complex. The first incision occurs at 5' end of the lesion by ERCC1-XPC complex with interacts with XPA of the pre-incision complex. Following this, synthesis of the repair strand occurs by recruitment of pol **δ**/**κ**/**ε**, starting at the incised 5' end. Then, a 3' incision occurs corresponding on the strand by XPG, while the synthesis of the new strand continues. Thus, the repair strand synthesis aided by DNA polymerases pol **δ**/**κ**/**ε** proceeds until the 3' end incision position and further the nick is sealed by ligation using DNA ligase I/III**α**.

1.2.4 Mismatch repair (MMR) (89):

MMR is triggered to repair the misincorporation of nucleobases, insertions/deletions (indels) that happen during DNA replication and recombination in living cells. It is a highly conserved pathway in eukaryotes. The eukaryotic MMR pathway (90) is complicated, and the proteins recruited depends on the short and long indels present in the daughter strand synthesized after the process is completed. MMR is tightly associated with the DNA replication and is highly active during the S-phase of the cell cycle. Deficiency of the MMR repair proteins will result in persistence and replication of mutations and these will lead to gene expression alterations. It was reported that their deficiency leads to colon, endometrial, ovarian and stomach cancers in mammals.

In this repair process (91, 92), the proteins MutS**α**/MutS**β** recognizes the small (~3 nucleotides) and large (~13 nucleotides) base mismatches/indels. MutS proteins bind at the mismatch site by PCNA/RFC complex, then recruits MutL**α** protein, which is a heterodimer with MLH1 and PMS2 subunits, to form a ternary complex. The endonuclease activity of the PMS2 is triggered to

translocate towards the 5' end of the mismatch and make a nick on the strand. Following this, EXO-I enzyme is recruited at the 5' nick and excises the nucleotides by progressing towards the 3' end, a few nucleotides past the mismatch site. The repair strand is then synthesized by recruiting pol **δ** at the 5' nick to proceed to 3' end. The gap at 3' end is then ligated by DNA ligase I.

1.2.5 Homologous recombination (HR) and Non-homologous end joining (NHEJ) (93):

DSBs in eukaryotes are repaired by two pathways, HR and NHEJ, where NHEJ is the dominant pathway. Failure to repair DSBs is fatal thereby leads to apoptosis and senescence of cells. Both pathways occur during the cell cycle, where HR occurs only during S and G2 phase of cell cycle and NHEJ occurs at G1 phase. Both of these pathways are important in the T-cell and B-cell development, immunoglobulin V(D)J combination.

In this repair process (94, 95), the initial step is recognition of DSB and to bind *Ku* heterodimer (70/80) on the 5' and 3' sides of the break. *Ku* acts as a scaffold to recruit the necessary factors and enzymes for the repair process. These are DNA-PKs, XRCC4, XLF, APLF, DNA ligase IV to form a stable complex with existing *Ku*-DNA which covers the area of DSB. DNA-PKs, then auto phosphorylate and releases from the complex producing a nick. The nick is then sealed by DNA ligase IV present in the complex. Followed ligation, remaining factors are released upon the ubiquitination of *Ku*. There exists an alternative pathway for more complex DNA damage which is ATM-dependent and requires Artemis nuclease to mediate the repair which is not discussed here.

1.3 TRANSLESION SYNTHESIS:

As mentioned in the previous sections, the DNA damage in the cells is most definitely be repaired by the various DNA repair mechanisms. But few of the lesions are inefficiently repaired by the cell. An alternative mechanism is adapted by the cells in order to survive, termed as translesion synthesis (TLS) (96, 97). The specialized polymerases called TLS polymerases bind at the stalled replisome to traverse the DNA damage and continue with the replication process. These TLS polymerases are of low-fidelity, hence may incorporate wrong nucleobases opposite the damage site inducing mutations, thus resulting in the error-prone replicative bypass. Irrespective of this, the DNA damage-tolerance mechanism is important for the cell survival and development of altered traits in the organisms (81). On the other hand, it is primarily responsible for mutagenesis and carcinogenesis. TLS is conserved from prokaryotes such as *E. coli* to mammals including humans (96).

Mechanism (97, 98):

TLS is triggered when the replisome encounters a damaged base and gets stalled. In normal replication process, PCNA along with PIP factor clamps/carries the high-fidelity replicative DNA polymerases **δ**/**ε** on lagging/leading strands respectively. At the stalled replisome, the polymerase switch from replicative to TLS polymerases is necessary. PCNA then gets ubiquitinated using proteins *Rad 6* and *Rad 18*, which recruits TLS polymerase/s to incorporate a complementary base opposite the damaged site and further extends a few bases. After the bypass of the damaged site and further extension, the polymerase switch was reversed as a result of deubiquitylation of PCNA, and replicative DNA polymerases will continue the replication. This reversal is important to reduce the misincorporation of bases opposite undamaged bases in the DNA replication process as TLS

polymerases have low fidelity. However, it is due to their wider enzyme catalytic sites as oppose to pol **δ**/**ε**, that they play an important role in the bypass of modified bases.

1.4 TLS POLYMERASES:

Five human TLS polymerases have been discovered, and they belong to Y-family (pol **η**, **κ**, **ι**, Rev1) and B-family (pol **ζ**) (99). TLS polymerases possess wider catalytic sites which can accommodate distorted and bulkier nucleobases, than the tight sites of replicative polymerases. However, they result in the misincorporation of bases due to lower fidelity, as they lack 3'->5' proofreading activity after the replication (96). Irrespective of regulated transcriptional processes, these polymerases may extend their replication beyond the damaged site and result in unwanted mutagenesis. TLS polymerases are conserved in evolution and the process occurs in *E. coli, S. cerevisiae* and *H. sapiens.* The different types of polymerases in humans and their roles are described below (100).

1.4.1 Polymerase η:

Polymerase **η** belongs to Y-family and is translated from gene *Rad 30A* (in humans) and *Rad 30* (in yeast). It plays an important role in the bypass of the UV-induced lesions (CPDs and 6-4 PPs) (101). Pol **η** possess broader active site which accommodate both dTs of CPD lesion and are bypassed in an error-free manner by incorporating dA complementary to it. The extension is also completed by pol **η** beyond the lesion site up to 3 bases (102). This ability of pol **η** to accommodate two bases facilitates the bypass of tandem lesions, such as ICLs at G-G sites formed by the therapeutic agent, cisplatin (103). In the bypass of 6-4 PPs, pol **η** can only accurately bypass one of the bases and a second polymerase, pol **ζ** is required to complete the bypass and further

extension (104-106). It is reported that pol **η** also plays an important role in error-free bypass by incorporating dC, opposite the oxidative DNA damage, 8-oxo-dG and its extension is facilitated by pol **ζ** (107, 108). Deficiency or mutation in pol **η** sequence, results in the genetic disorder xeroderma pigmentosum variant (XP-V), due to which the individual is susceptible to the UV-sunlight induced skin cancer (109).

1.4.2 Polymerase ι:

Polymerase **ι** plays an important role in the bypass of oxidative DNA damages (110). It is translated from the gene *Rad 30B* in humans and is absent in yeast (111). A unique trait of pol **ι** is the replication may proceed both in error-free or error-prone manner. This nature of pol **ι** bypass is dependent on the conformation of the lesion in its unique active catalytic site. If a *syn* conformation is adapted, it will result in hoogsteen-base pairing with the incoming nucleobase (112). With the exception of 8-oxo-dG bypass, where the lesion adapts a *syn* conformation in the pol **ι** active site, it results in a Watson-Crick base pairing with the incoming dC base (110). There also exists an asymmetric fidelity between damaged bases, dA and dT, where pol **ι** incorporates dT (correct base) and dG (incorrect base) respectively during the TLS (113). Pol **ι** can also bypass some of the tandem lesions, when the damages are present in closer proximity to each other. Pol **ι** and pol **η** are reported to have a role in the bypass of CPDs *in vivo*, which is proved by the resulted accelerated UV-induced tumors in mice (114). In few cases, pol **ι** also participates in the short-patch BER process replacing pol **β** function, where pol **ι** possesses dRP lyase activity and interacts with the scaffold protein XRCC1 (115). Due to its error-prone nature, pol **ι** is also responsible for the occurrence of somatic hypermutations in the antibody genes *in vivo (97)*.

1.4.3 Polymerase κ:

Polymerase **κ** plays an important role in the error-free bypass of the various bulky N^2-dG-linked adducts and ICLs. Pol **κ** is conserved in bacteria and humans but not in yeast (100). The active catalytic site of pol **κ** is reported to possess an N-terminal extension (N-clasp) which can recognize the accommodation of bulky lesions in the minor groove of DNA (116). It is also reported that the dinucleotide lesions block the pol **κ** bypass because, the active site of pol **κ** can fit only one Watson-Crick base pair (116). Pol **κ** aids in the extension of DNA past the damaged site with high fidelity, in association with other TLS polymerases like pol **ι**, pol **ζ** which participates in the lesion site bypass (117). For example (117), in cisplatin-ICL adduct (Pt-GG), *in vitro* primer extension studies proved that pol **κ** was unable to bypass the first encountered 3'-dG but can bypass the second 5'-dG. Hence, base incorporation opposite 3'-dG is aided by pol **η** and then pol **κ** incorporates dC opposite 5'-dG. Pol **κ** is also hypothesized to participate in the NER process, particularly associates with pol **δ** in the gap filling step after the excision step in NER. However, this association could lead to high mutation rate in the repaired strand (118).

1.4.4 Polymerase Rev1:

Rev1 polymerase is translated from gene *Rev1* in both humans and yeast. Rev1 acts as both catalytic and non-catalytic forms in TLS mechanism (111). The catalytic role of Rev1 is to incorporate dC opposite limited types of dG adducts and abasic sites. TLS bypass by Rev1 proceeds in a unique manner where the damaged base (dG) is displaced from the double helix and the incoming dC is paired with the Arg-324 moiety of Rev1 (119). Hence, the polymerase is also termed as template-independent dC transferase. It is also involved in the formation of DNA secondary structures, triplex repeats and G-quadruplexes (120).

The non-catalytic role of Rev1 polymerase is to coordinate interactions with PCNA, ubiquitin and other TLS polymerases (121). In yeast cell mutagenesis, Rev1 interacts with Rev3 subunit via Rev7, pol31, pol32 accessory subunits of pol **ζ** to promote the error-prone extension bypass (122). In human cells, Rev1 acts as a scaffold at the stalled replication fork and recruit Y-family polymerases for the base incorporation step and pol **ζ** for the extension step of TLS (111).

1.4.5 Polymerase ζ:

Polymerase **ζ** plays a major role in the TLS and damage-induced mutagenesis in yeast and human cells. In humans, pol **ζ** comprises of four subunits, catalytic Rev3, accessory subunits Rev7, polD2, polD3, hence may be represented as **ζ** 4 (97, 123). Rev3 is large subunit with > 3000 residues and is twice as the size of the yeast homolog Rev3p. Yeast also contains **ζ** 4 with Rev3-Rev7-pol31-pol32 subunits (124). Rev3 is translated from *Rev3L* gene in humans. Rev7 is an important non-catalytic subunit and links the Rev3 of pol **ζ** to Rev1 polymerase which promotes the TLS replication bypass of damaged DNA (97). Mainly, it is reported that pol **ζ** -Rev1 complex play an important in the DSB repair (97). Pol **ζ** mainly involves in the error-prone lesion bypass as it lacks the proofreading exonuclease activity. Pol **ζ** involves in the extension step of TLS, as it is inefficient in the base incorporation opposite the damaged base (108). For instance, it is reported (125) that in the TLS bypass of the cisplatin adduct, pol **η** participates in the base incorporation and pol **ζ** in the strand extension step, before the polymerase switch. It is reported from the human and mice cell studies that mutation in *Rev3L* gene resulted in the embryo lethality and growth defects (126). Hence, pol **ζ** is important in the normal cell proliferation and development of embryos in mammals.

1.5 Overview of Dissertation:

The goal of the dissertation was to investigate the replicative bypass of the damaged DNA adducts in human embryonic kidney (HEK 293T) cells. Three DNA adducts, bulky DNA-peptide cross-link, a tandem lesion containing oxidative abasic site, 2-deoxyribonolactone and thymine glycol, oxidative stress induced formamidopyrimidines were studied. A site-specific approach was employed to understand the mutagenicity and cytotoxicity of these adducts in human cells. For this purpose, three single stranded recombinant vectors were constructed with these adducts and replicated in HEK 293T cells along with selective single/double/triple knockdown or knockout of TLS polymerases from the cells. These polymerase deficient cells were obtained either by siRNA-induced gene silencing or CRISPR-Cas9 mediated gene editing techniques. Further, the mutational screening was achieved by performing Southern blot hybridization followed by DNA sequencing analysis. The biological implications of these adducts were studied independently in the subsequent chapters which were outlined below.

Chapter 1 focusses on the introduction of various concepts that facilitates in the understanding of DNA damage and its replication in the cells. A broad overview of types of DNA damages and its various sources were discussed. Detailed review of DNA protein cross-links, abasic sites and oxidative stress induced DNA adducts was included. Unique DNA repair mechanisms which recognize and eliminate various adducts were described. Finally, the adducts which were immune to repair, were replicated by a DNA damage tolerance mechanism termed as translesion synthesis (TLS) was explained. Further, TLS is mediated by various polymerases, with adduct specific recognition criteria were also discussed.

Chapter 2 focusses on the materials and methods employed in the study. Various chemicals, enzymes and reagents used were mentioned. A detailed methodology of the techniques involved in the recombinant DNA technology, siRNA mediated transfection, *in vitro* primer extension, nucleotide incorporation assays were discussed. A comprehensive protocol description was included for the Southern blot and western blot hybridizations along with denaturing polyacrylamide and agarose gel electrophoresis. The sequences of probes and primers designed and employed in various procedures were reported. This explicit review of the techniques will enable the reader to understand the choice of these methods and its importance in the conclusions of the study.

Chapter 3 focusses on the mutagenicity and cytotoxicity effects of epigenetic mark, 5-formylcytosine mediated DNA peptide cross-links (DpC) in human cells. A detailed explanation of the formation and synthesis of adduct was included. From the calculated mutation frequencies, it was revealed that the predominant targeted site mutations were C->T and C-deletions. From the TLS efficiencies obtained, it was evident that the predominant bypass of DpC is through TLS in an error-prone manner. However, from the *in vitro* primer extension assays, it was observed that replicative polymerases were as well able to bypass the damage in an error-free manner, albeit very slowly.

Chapter 4 focusses on the compare and contrast study of a tandem lesion with that of its corresponding isolated lesions, in terms of its TLS efficiencies, nucleotide incorporations and deletions during replication. The tandem lesion (LTg) studied include oxidized abasic site 2-deoxyribonolactone (L), positioned 5' to thymine glycol (Tg) and its TLS efficiencies were

compared with isolated lesions, L and Tg separately in human cells. It was obvious that the cytotoxicity during tandem lesion replication was greater than their isolated lesions. It was also revealed that, all the lesions were replicated through TLS mechanism, mainly with pol **ζ** coordinated with other TLS polymerases and that Tg is not a strong replication block in human cells. It was evident from the mutational data that, the predominant nucleotide incorporation opposite L in both tandem and isolated positions was deoxyadenine. This proves that the widely reported 'A-rule', nucleotide incorporation opposite abasic site was in fact followed in the TLS bypass of these oxidative abasic lesions.

Chapter 5 focusses on the mutagenicity and cytotoxicity of oxidative stress induced formamidopyrimidine - deoxyguanine adduct, Fapy.dG in human cells. A detailed scheme for the formation of these adducts along with their **α** and **β** conformation anomers were shown. Predominantly, Fapy.dG adducts exists as a **β** anomer in DNA, hence an oligonucleotide adduct was synthesized for the experimental purposes, was described. From the single TLS polymerase knockout data, it can be hypothesized that polymerases **ζ**, **ι**, **κ** play important role in the error-prone replication. Future work on double knockdown experiments will reveal the biological consequences of Fapy.dG replication in human cells.

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CHAPTER – 2

MATERIALS AND METHODS

2.1 MATERIALS:

2.1.1 Enzymes:

All the enzymes used for restriction digestion, phosphorylation, ligation, scaffold removal, proteolysis reactions were *EcoR*V, T4 polynucleotide kinase, T4 DNA ligase, T4 DNA polymerase, M13 helper phage, uracil DNA glycosylase, proteinase K, exonuclease III and their corresponding buffers were purchased from New England Biolabs (Beverly, MA). **γ**-^{32}P ATP used for 5' end oligonucleotide labeling, and autoradiography was purchased from Perkin Elmer Life Sciences (Shelton, CT).

2.1.2 Chemicals:

The chemicals and reagents of commercial grade used for preparation of gels, buffers and wash solutions were tris-base, sodium chloride, boric acid, acrylamide, N, N'-methylene bis-acrylamide, disodium EDTA, TEMED, Ammonium persulfate, urea, agarose, HEPES sodium salt, sodium hydroxide, sodium citrate dihydrate and media components for bacterial growth include tryptone, yeast extract, agar were purchased from Fisher scientific (Agawam, MA). Oligonucleotide hybridization buffer (PerfectHyb™ 1X buffer) was purchased from Sigma-Aldrich (St. Louis, MO). Phenol-chloroform-isoamyl alcohol (25:24:1) solution for plasmid purification was purchased from Invitrogen (Carlsbad, CA).

2.1.3 Reagents and cells:

Bacterial competent cells *E.coli* DH10B, DH12S for electroporation, mammalian cell culture media components such as Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), non-essential amino acids (NEAA), penicillin-streptomycin solution, transfection components such as Opti-MEM reduced serum medium, Lipofectamine™ 2000 were purchased from Invitrogen (Carlsbad, CA). Synthetic siRNA duplexes used in the inactivation of pol **η**, pol **κ**, pol **ι**, pol **ζ** as well as the DNA extraction kit (cat # 51306), one step RT-PCR kit (cat # 210210), QuantiTect SYBR green RT-PCR kit (cat # 204243), RNeasy plus kit (cat # 74134) to isolate DNA, from HEK 293T cells were purchased from Qiagen (Valencia, CA).

HEK 293T cells were obtained from ATCC (Manassas, VA). The unmodified nucleotides (controls), scaffolds, oligonucleotide probes for hybridization and primers for sequencing were purchased from Integrated DNA technologies (Coralville, IA). Costar spin-X tubes from Corning (NY), Sep-Pak C18 cartridges from Waters corporation (Milford, MA), Amicon ultra filtration units from Millipore Sigma (Burlington, MA) were obtained and employed in plasmid and oligonucleotide purification.

2.2 METHODS:

2.2.1 Purification of oligonucleotides using polyacrylamide gel electrophoresis (PAGE):

Modified oligonucleotides (adducts), unmodified (controls), scaffolds were purified using 16 %-20 % denaturing polyacrylamide gel electrophoresis (PAGE). The gel was formed by free radical initiated co-polymerization of acrylamide and bis-acrylamide along with 7 M urea, aided by 25 % ammonium persulfate, catalyzed by TEMED in 1X TBE (tris-borate-EDTA) buffer. Samples in water, up to 30 micrograms were mixed with the formamide dye (containing xylene cyanol and bromophenol blue dyes) and loaded onto the gel and run at 2000 V for 5 h to resolve the bands. The bands were then visualized under UV light and the desired gel fragments were excised. The oligonucleotide samples were back extracted from gel bands using 1X TE (tris-EDTA) buffer and co-star spin-X columns to separate the gel fragments. The filtrate with the desired oligo was further desalted using Sep-Pak C18 cartridges and were eluted in 60 % Acetonitrile solution. The acetonitrile was removed, and the samples were concentrated using hispeed-vacuum system and the resulting oligos were quantified using nano-drop™ spectrophotometer. To check the purity of the oligonucleotides extracted, about 100 nanograms of the oligo was radiolabeled at 5' - end using **γ**- ^{32}P ATP and was run on the 16 % PAGE gel under same conditions. After the run, the bands were visualized and analyzed by autoradiography.

2.2.2 Plasmid quantification using agarose gel electrophoresis:

The plasmid employed in this research was a circular, single stranded pMS2 of size 5137 bp. The quality and the quantity of the plasmid extracted from *E.coli* was verified using 1.1 % agarose gel electrophoresis. The gel was formed by polymerization of the agarose powder in 1X TBE when

heated. The DNA samples were mixed with the 6X loading dye (NEB) and were run on the gel for 3 h at 110 V. After the run, the gel was stained with ethidium bromide (10 mg/ml) and then allowed to de-stain in water to remove background. Then the bands were visualized under Bio-Rad UV gel documentation analysis system and the intensities of the desired bands were obtained. Their intensities were then compared to the standard to calculate the unknown plasmid concentration as follows:

Unknown plasmid concentration (µg/µl) = (unknown plasmid band intensity / known plasmid band intensity) * known plasmid concentration. (µg/µl)

2.2.3 Preparation of single stranded pMS2 plasmid:

Single stranded circular pMS2 plasmid of 5137 bp was prepared from *E.coli* JM109 using M13 helper phage which was reported in (1). The plasmid contains SV40, f1, ColE1 origins of replication which aids in its replication in both mammalian and *E.coli* cells. The vector also contains neomycin and ampicillin resistant genes which helps in the selection of transformants containing pMS2 vector.

Firstly, electroporate 50 nanograms of double stranded pMS2 plasmid in *E.coli* DH12S cells under conditions-1.8 kV, 25 µF and 200 Ω. To the electroporated culture, 1 ml of pre-warmed SOC media was added and was revived by shaking at 230 rpm, 37 °C for 1 h. The revived culture was then plated on 1X yeast-tryptone (YT) media plates containing ampicillin and incubated for 16 h at 37 °C to obtain single colonies. Next day, ~100 colonies were scraped and sub-cultured in 2 liters of 1X yeast-tryptone liquid media containing ampicillin (100 µg/ml) and 1 ml of M13 helper phage. After 2 h of shaking at 250 rpm, 37 °C, add 15 ml kanamycin (5000 µg/ml) and continued shaking for up to 20 h. This phage-infected culture was centrifugated at 10 k rpm for 20 min at 4 °C twice,

to separate the pellet. To the supernatant, add 4 % (w/v) of PEG-8000 and 3 % (w/v) sodium chloride and stirred slowly at 4 °C to separate the phage particles. After 2 h of stirring, the solution was centrifugated at 10 k rpm for 30 min at 4 °C to precipitate the phage particles. The precipitate was resuspended in 1X tris-EDTA (TE) buffer and incubated with proteinase-K, 10 % SDS for 16 h at 42 °C to precipitate the lysed phage proteins. DNA was back extracted from the mixture using phenol-chloroform-isoamyl alcohol solution. Briefly, the phenol solution was added 1:1 ratio to the DNA mixture and centrifugated at 14000 rpm for 20 min at room temperature and was repeated thrice. The top layer contains the plasmid and bottom layer contains the lysed proteins. Hence the top layer was collected and purified using 100 K MWCO Amicon ultra centrifugal filtration or by ethanol precipitation using ammonium acetate. The obtained plasmid DNA pellet was resuspended in water and quantified using 1.1 % agarose gel electrophoresis.

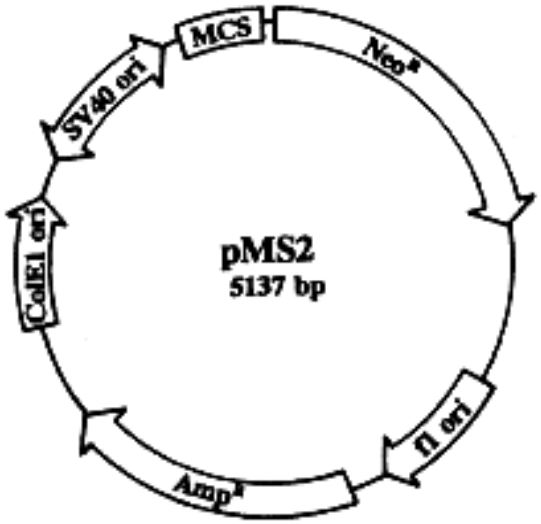
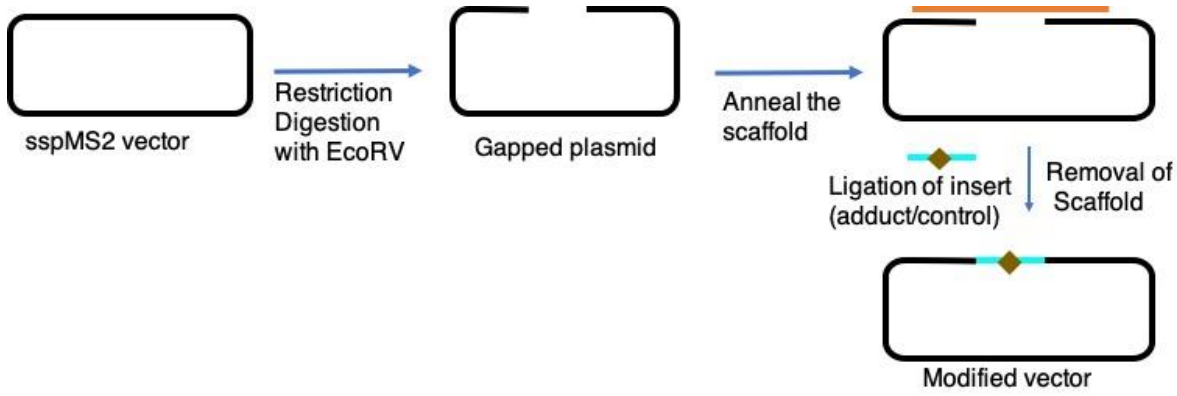


***FIGURE 2.1* - Single stranded pMS2 vector with ColE1, SV40, f1 origins of replication and MCS (multiple cloning site) containing restriction site.**

2.2.4 Construction of modified pMS2 vector with site specific incorporation of adducts:

25 micrograms of single stranded pMS2 plasmid contains a restriction site (5' GAT-ATC 3') which was digested by *EcoR*V enzyme to yield a gapped plasmid. A 62-mer PAGE purified scaffold, designed to be complementary to the adduct and the flanking sequences of pMS2 plasmid, was annealed at 75 °C to the gapped plasmid, allowed to cool down to room temperature. Then, a 30-fold PAGE purified adduct oligonucleotide/control was phosphorylated at 5' end using T4 polynucleotide kinase enzyme at 37 °C for 1 h and then ligated to the scaffold-annealed pMS2 plasmid using T4 DNA ligase enzyme at 16 °C for 18 h to form phosphodiester bonds with the plasmid. Following ligation, the scaffold was removed by treating with uracil-DNA glycosylase and exonuclease III for 16 h at 16 °C. After each step, an aliquot of the plasmid was run on 1.1 % agarose gel to confirm the completion of the reaction. Following de-scaffolding step, the modified constructs were extracted using phenol-chloroform solution and ethanol precipitation. The constructs were quantified using 1.1 % agarose gel electrophoresis.



SCHEME 2.1: Protocol for the construction of recombinant vector with the oligonucleotide insert (adduct/control).

2.2.5 Transfection of recombinant vector in human cells:

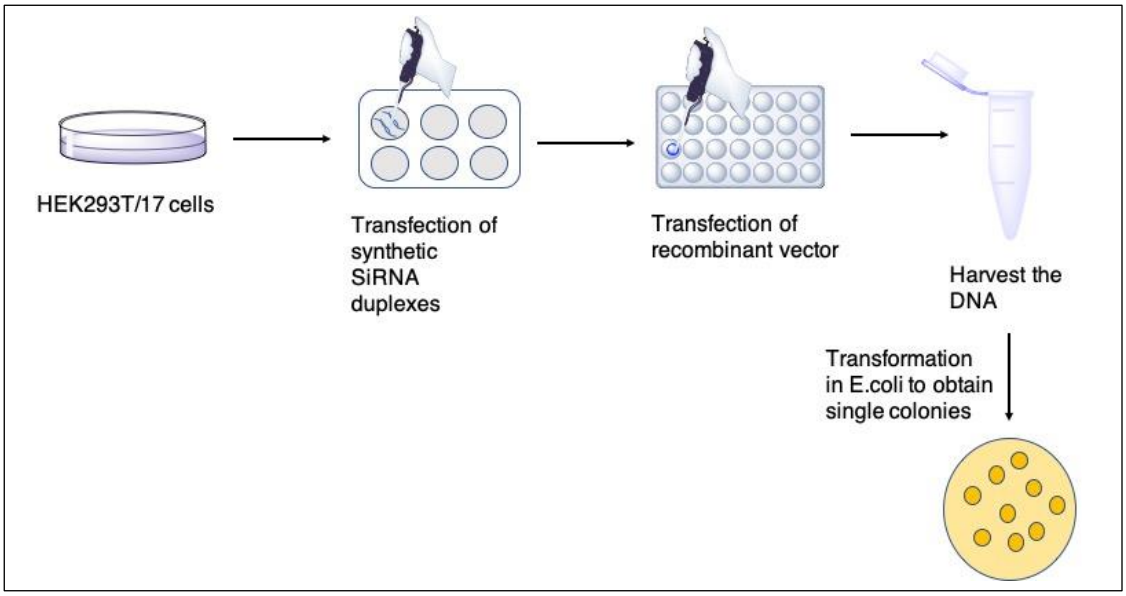
HEK 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), 10 % Fetal Bovine Serum (FBS), 2.4 % non-essential amino acids (NEAA) and maintained at 37 °C, 5 % CO_2. A 24-well plate was prepared and allowed to grow until 50 % confluency. Then, the adducted vector along with the control was transfected using lipofectamine-2000 and opti-MEM reagents. After 24-48 h, the cells attained 100 % confluency were harvested using **β**-mercaptoethanol and RLT lysis buffer (Qiagen), then stored at -80 °C until next step.

2.2.6 Translesion synthesis (TLS) polymerase knockdown using siRNA transfection in human cells:

HEK 293T cells were cultured and maintained under same conditions as mentioned above. A 6-well plate was prepared and allowed to grow until 50 % confluency, 100 picomoles of first shot of synthetic siRNA duplex against the specific polymerase was transfected using lipofectamine-2000 and opti-MEM reagents. After the cells have reached full confluency, they were sub-cultured into 24-well plates and allowed to grow until 50 % confluency. Followed by co-transfection of adducted vector/control and 30 picomoles of second shot of siRNA duplex. After 24-48 h, when the cells reach full confluency, they were harvested using **β**- mercaptoethanol and RLT lysis buffer (Qiagen), then stored at -80 °C until next step.

Gene	siRNA (sense) sequence
pol H	5' – GGUUGUGAGCAUUCGUGUATT - 3'
pol K	5' – CUGUGAGUAAAGAGGUUAATT - 3'
pol I	5' – GCCUCAUACAGUGAGAUUATT - 3'
pol Z	5' – GCAAUUUUGAACCUUAUGGTT - 3'
Rev 1	5' – CGGUGGAAUCGGUUUGGAATT - 3'

TABLE 2.1: Represents the specific siRNA sequences designed and used in the single/double/ triple knockdown of TLS polymerases.



SCHEME 2.2: Represents the siRNA induced knockdown of TLS polymerases and transfection of recombinant vector in HEK 293T cells. Followed by transformation of harvested DNA in E.coli DH10B competent cells.

2.2.7 Construction of TLS polymerase knockout cell lines:

The single TLS polymerase knockouts were performed in HEK 293T cells using commercially available specific CRISPR/Cas9 knockout plasmids for pol **η**, pol **ι**, pol **κ**, pol **ζ**. These plasmids were transfected in cultured HEK 293T cells using the same procedure as described above.

Following transfection, single cells were sorted into 96-well plates using a BD FACSAria II flow cytometer. After clonal expansion, the monoclonal cell cultures were screened by western blotting for the protein expression levels. Similarly, the double knockout cell lines (pol **η**/**κ**) were constructed.

2.2.8 Total RNA isolation and quantification:

When the siRNA knockdown of TLS polymerases was performed in HEK 293T cells, it was important to note the efficiency of knockdown from the total RNA isolated by using RNeasy plus kit (cat # 74134). Total RNA was isolated according to the manufacturer's recommended protocol. After 72 h of the siRNA transfection, HEK 293T cells were lysed by the addition of RLT buffer with **β**-mercaptoethanol and collected. The lysed cells (lysate) was homogenized by vortexing for 1 min. Genomic DNA (gDNA) eliminator spin columns were used to isolate DNA from the lysate by centrifugation. To the filtrate, equal volume of ethanol was added. This mixture was passed through the RNeasy Min Elute spin column, to bind the RNA to the column by centrifugation. The column was washed with the wash buffers. RNA was eluted in water and quantified. Total RNA quantification was performed by Nanodrop 2000c spectrophotometer, by adapting a convention in terms of absorbance. 1 unit of absorbance at A260 is equivalent to 40 μg/ml of RNA. Purity of the RNA can be tested by the 260/280 ratio which should ideally be 2.0, although 1.9 and 2.1 are acceptable.

2.2.9 Reverse transcription – polymerase chain reaction (RT-PCR) analysis:

cDNA synthesis was performed by RT-PCR, using one step RT-PCR kit (cat # 210210) according to manufacturer's protocol and literature (2). Briefly, 100 nanograms of total RNA isolated was

used as template, GAPDH as control, primers specific to the TLS polymerases were designed and listed below. Master mix was prepared by adding the primers, dNTP mix, enzyme mix buffer (Taq polymerase, Mg^{+2}, K^{+}, NH_4^{+}). Then the mix was distributed to the template containing tubes used for the thermocycler. First steps were to perform reverse transcription programmed at 30 min at 50 ºC and PCR activation at 15 min at 95 ºC. The amplification steps - denaturation, annealing and extension were programmed at 94 ºC for 30 s, 55 ºC for 45 s, 72 ºC for 60 s respectively. 26 cycles were performed for pol **η**, pol **ι**, pol **κ**, Rev1; 32 cycles for pol **ζ**; 24 cycles for GAPDH. The RT-PCR products were run on the 2 % agarose gel at 100 V for 3 hrs.

Gene	Primer	Primer sequence
pol H	Forward	5' – CTACTCGGGAACAGGTACAATG - 3'
	Reverse	5' – GTGGGAGCAGTAAGAGATGAAA - 3'
pol K	Forward	5' – AGCTGTGAGTAAAGAGGTTAAGG - 3'
	Reverse	5' – TGAACCTAGACCCAAGGAGATA - 3'
pol I	Forward	5' – ATTAGGGACAGGAAATTATG - 3'
	Reverse	5' – CAATGTCAGAAGGGAAAAG - 3'
pol Z	Forward	5' – GAGAGAAGCACAAGTCCCATAAA - 3'
	Reverse	5' – CTAATGGGCTCTGCTGTAGATC - 3'
Rev 1	Forward	5' – AATCTACCAGGAGTTGGA - 3'
	Reverse	5' – GTGGAAGGGTTCAGATTAG - 3'
NC	Forward	5' – ACCACAGTCCATGCCATCAC - 3'
(GAPDH)	Reverse	5' – TCCACCACCCTGTTGCTGTA - 3'

TABLE 2.2: Primers used for RT-PCR quantification of siRNA knockdown of TLS polymerases.

2.2.10 Real-time quantitative RT-PCR:

Another method for synthesis of cDNA from total RNA as well as to quantify was obtained by performing real-time quantitative RT-PCR. The instrument used was Bio-Rad CFX-96 Real-Time PCR detection system using QuantiTect SYBR Green RT-PCR kit (cat # 204243) and protocol according to the manufacturer's instructions. Gene expression was quantified using cycle threshold method ($2^{-\Delta\Delta C}T$). SYBR Green PCR master mix was provided and the primers were added. This mixture was distributed to the PCR tubes containing the template/control. The cycles were programmed as follows: reverse transcription at 50 ºC for 30 min, PCR activation step at 95 ºC for 15 min for one cycle each, amplification steps-denaturation at 94 ºC for 15 s, annealing at 55 ºC for 30 s, extension at 72 ºC for 30 s for 40 cycles.

2.2.11 Western blot analysis:

After the siRNA polymerase knockdown (KD)/CRISPR-Cas9 polymerase knockout (KO) was performed, the gene silencing was confirmed by western blot analysis as per literature (2). After 48 h of the KD/KO, the cells were lysed and collected in ice cold RIPA buffer containing protease inhibitor cocktail. The cell suspension was incubated for 1 h on ice and centrifuged at 10000 rpm for 15 min at 4 ºC. The concentration of the proteins in the supernatant (whole cell lysate) was determined by Bradford protein assay. Whole cell lysates were boiled in the loading buffer, containing 250 mM tris (pH 6.8), 5% SDS, 30 % glycerol, 20% **β**- mercaptoethanol, bromophenol blue. The proteins are resolved by 5 %-7 % SDS-PAGE gel, run for 2 h. The gel bands were transferred onto PVDF membranes and blocked with 5 % milk. These membranes were incubated with polymerase specific primary antibodies, with human **β**-actin antibody as control. Then the complexes were incubated with HRP-conjugated goat anti-rabbit, secondary antibodies. A

substrate, Pierce ECL western blotting substrate was employed for the purpose of electrochemiluminescence detection and the images were developed using Bio-Rad Personal molecular imager (PMI) phosphorimager.

2.2.12 Double stranded DNA extraction from HEK 293T cells:

Harvested cells after transfection stored at -80 °C were thawed rapidly at 37 °C. The samples were treated with the Qiagen columns (Cat # 51306) for the extraction of the double stranded DNA. Briefly, the columns with the samples were spun at 10000 rpm for 1min at room temperature to trap the DNA to the column. Then the column was washed with wash buffer-1 and wash buffer-2 at 14000 rpm for 2 min. The DNA was eluted from the column with elution buffer (pre-heated at 70 °C) at 10000 rpm for 1 min.

2.2.13 Electroporation of double stranded DNA (dsDNA):

The dsDNA extracted from the previous step was transformed into *E.coli* DH10B electrocompetent cells and the culture was revived in 1 ml SOC media by shaking at 37 °C, 230 rpm for 1 hr. This culture was plated onto 1X YT plates containing ampicillin and incubated for 16 h at 37 °C to obtain single colonies. Bacterial single colonies were picked and grown in the liquid 1X YT media containing ampicillin for 4 h at 37 °C, until log phase.

2.2.14 Replication and fixation of double stranded DNA onto Whatman filters:

The sub-cultured bacteria were blotted onto Whatman filter paper using replicator, were allowed to grow on the filter paper for 18 h at 37°C. These filters were washed with a series of wash

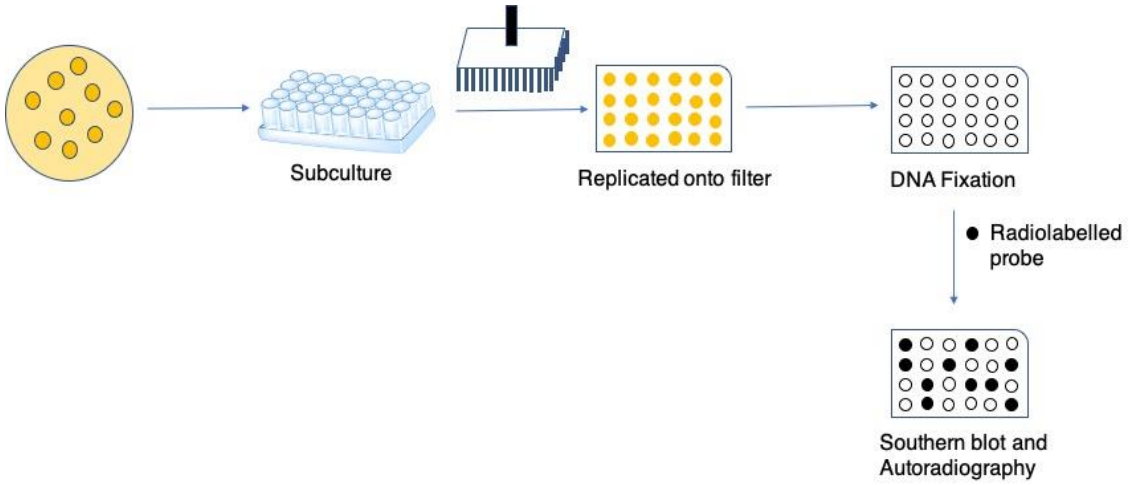
solutions which lysed the bacteria and released the DNA. Briefly, the filters were first treated with 0.5 N NaOH for 5 min, followed by 0.5 M tris-HCl (pH 7.7) for 4 min. Then the filters were treated with 2X SSC (saline sodium citrate) for 2 min and finally with 200 proof ethanol for 1 min. After each wash, the filters were blotted to remove the excess solution and then briefly air-dried. The released DNA was then fixed onto the Whatman filters by heating up to 80 °C until they were absolutely dry.

2.2.15 Mutational screening by Southern blot hybridization:

Three oligonucleotide probes were designed with the sequences complementary to the adduct/control containing DNA replicated in HEK 293T cells. They were named as left probe (LP), right probe (RP) and wild type probe (WT) as shown below. These probes were radiolabeled using **γ**-^{32}P ATP at 5' end using T4 polynucleotide kinase enzyme at 37 °C for 1 h. Following this, the radiolabeled probes were allowed to hybridize to the adduct/control containing DNA, fixed on the filters, in hybridization buffer for 10 h at their specific melting temperature (49 °C/50 °C). Following hybridization, the filters were washed at the same hybridization temperature using pre-heated 2X SSC buffer, twice to remove the background. The filters were then exposed to the screen to capture the image using Bio-Rad personal molecular imager (autoradiography).

In the image obtained, the presence of the signals at the specific transformants reflects the binding of a particular probe to the DNA. To screen the mutants, first, the transformants that bind to both RP and LP were considered as those that contain the adduct/control DNA and the others were excluded. Then, the transformants that failed to bind to the WT but bound to RP/LP are identified and termed as mutants as the modification of the sequence has resulted in the failure of hybridization of probe. Hence, these mutants were sent for commercial sequencing analyses using

specific pMS2 primer designed to identify the specific position and the type of the mutation occurred.



SCHEME 2.3: Protocol for performing mutational screening by Southern blot hybridization followed by DNA sequencing analysis.

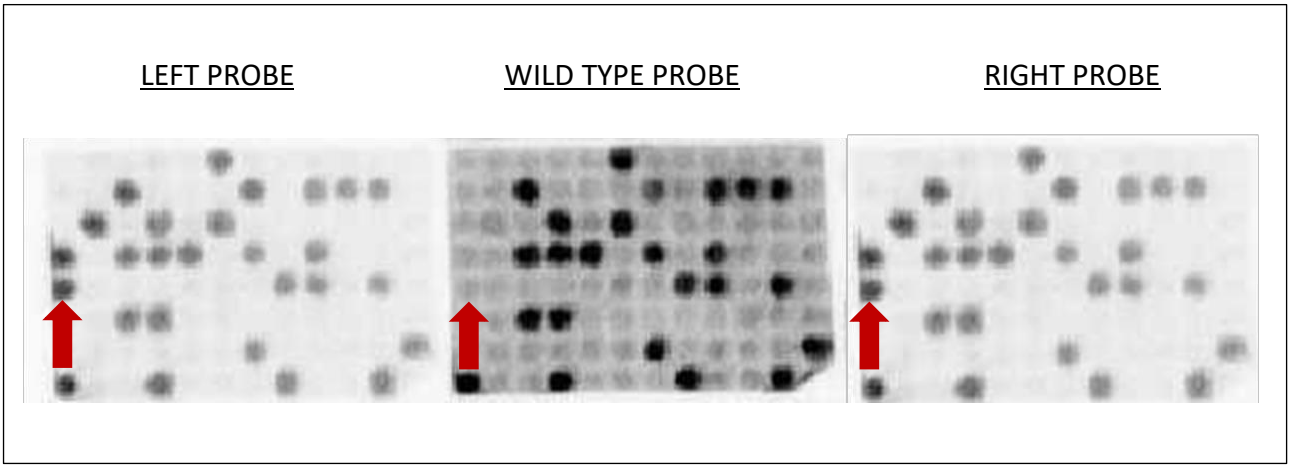


FIGURE 2.2: Represents a typical image obtained post-hybridization by autoradiography. The signals represent the hybridization of corresponding probe to the adduct/control. The red arrows represent a typical mutant.

Sequence of the adduct/control (Fapy.dG)	5' - GTG CGT GTT TGT – 3'
Left probe (LP)	5' – GGT ACC AGC GAT GT - 3'
Wild type probe (WT)	5' – TGT GCG TGTT TGT AT – 3'
Right probe (RP)	5' – TGT ATC GCT TGC AG – 3'
pMS2 primer used for DNA sequencing (reverse)	5' – AGA ACC TGC GTG CAA TCC -3'

TABLE 2.3: Sequences of probes designed specifically for an adduct/control (eg. Fapy.dG) for Southern blot hybridization with melting temperature at 49 ºC. A reverse primer designed for sequencing process of DNA extracted from mutant cultures.

2.2.16 TLS efficiency:

TLS efficiency of the adducts when replicated in the different kinds of cells was calculated to understand how knockout (KO) or knockdown (KD) or specific polymerases affect the replication. To achieve this, adducted pMS2 constructs were transfected along with the internal control (IC) constructs in 1:1 ratio. IC constructs are the unmodified constructs with altered sequence. After amplification in *E.coli*, the progeny was analyzed by Southern blot hybridization using oligonucleotide probes specific to adduct and IC. TLS efficiency was calculated as the percentages of the colonies from replication of the adduct to the IC.

TLS efficiency (%) = (# signals of adduct / # signals of IC) * 10

Sequence of Internal control	5'- AGG GTT TAC CCA GTC ACG ACG TT – 3'
IC probe	5' – TAC CCA GTC ACG ACG – 3'

TABLE 2.4: Represents the sequence of internal control used to calculate TLS efficiency and the corresponding IC probe designed for Southern blot hybridization.

2.2.17 Primer extension assay using human replicative polymerases:

Primers were radiolabeled at 5' end with **γ**-^{32}P ATP as described above. The DNA templates (adduct and control) were annealed with labeled primers. These primer-template complexes (1 pmol) were incubated with the mixture of 1 mM of four dNTPs. The buffer used in this assay with the composition of 25 mM tris/HCl (pH - 7.5), 8 mM $MgCl_2$, 5 mM DTT, 100 µg/ml BSA and 10 % glycerol. The polymerization reaction was initiated by addition of either 0.56 pmol of pol **δ** or pol **ε** to the primer-template solution. At certain time intervals (0 min, 5 min, 15 min, 30 min),

aliquots were collected, and the reaction was quenched by the addition of the gel loading buffer (20 mM EDTA in 95 % formamide, 0.05 % bromophenol blue, 0.05 mM xylene cyanol). The samples were then run on the 20 % denaturing PAGE gel at 80 W for 2 h to resolve the primer excision bands. The bands were then visualized using autoradiography.

2.2.18 Single-nucleotide incorporation assay:

Primers were radiolabeled at 5' end with **γ**-^{32}P ATP as described above. The DNA templates (adduct and control) were annealed with labeled primers. These primer-template complexes (1 pmol) were incubated with the mixture of 1 mM of four dNTPs. The buffer used in this assay with the composition of 25 mM tris/ HCl (pH 7.5), 8 mM $MgCl_2$, 5 mM DTT, 100 µg/ml BSA and 10 % glycerol. The reaction was initiated by the addition of a single dNTP (dATP, dTTP, dGTP, dCTP). At certain time intervals (0 min, 5 min, 15 min, 30 min), aliquots were collected, and the reaction was quenched by the addition of the gel loading buffer (20 mM EDTA in 95 % formamide, 0.05 % bromophenol blue, 0.05 mM xylene cyanol). The samples were then run on the 20 % denaturing PAGE gel at 80 W for 2 h to resolve the primer excision bands. The bands were then visualized using autoradiography.

2.3 REFERENCES:

1. Moriya, M. (1993) Single-stranded shuttle phagemid for mutagenesis studies in mammalian cells: 8-oxoguanine in DNA induces targeted G.C-->T.A transversions in simian kidney cells. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 1122-1126
2. Pande, P., Malik, C. K., Bose, A., Jasti, V. P., and Basu, A. K. (2014) Mutational analysis of the C8-guanine adduct of the environmental carcinogen 3-nitrobenzanthrone in human cells: critical roles of DNA polymerases η and κ and Rev1 in error-prone translesion synthesis. *Biochemistry* **53**, 5323-5331

CHAPTER – 3:

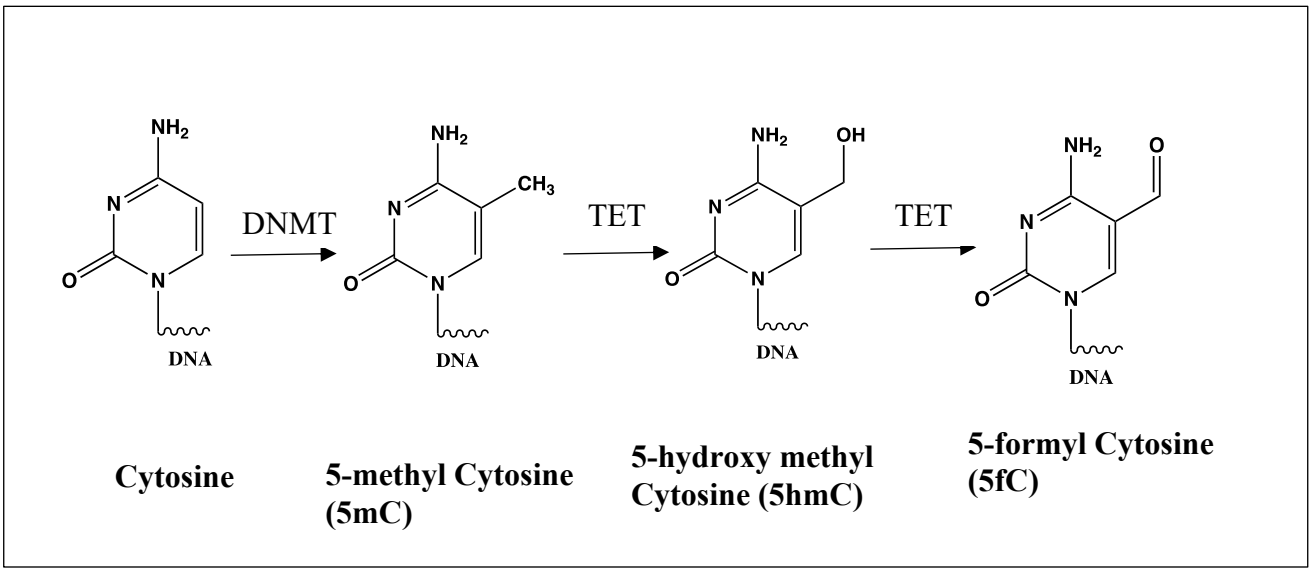
Error-prone replication of a 5-formylcytosine-mediated DNA peptide cross-link in human cells

3.1 INTRODUCTION:

DNA protein cross-links (DPCs) are formed when proteins covalently bound to DNA (1, 2). Their formation was first reported in 1960s (3). DPCs can be induced by exposure to exogenous agents such as anti-tumor drugs, transition metals and UV light or endogenously by cellular processes such as lipid peroxidation, histone demethylation, DNA replication, transcription, and DNA repair (1, 2, 4-9). They interfere with the chromatic architecture (10), DNA repair, block DNA replication and transcription. DPCs are toxic and induce mutations (1, 2, 4, 5, 11-13) when replicated, which leads to cancer and aging (14-17). Initial steps of DPC repair are presumed to involve proteolytic digestion of cross-linked protein into its peptide fragment. This small peptide fragment, termed as DNA peptide cross-link (DpC) can be repaired by NER (12, 18, 19) or if unrepaired, bypassed by TLS mechanism (18, 19). Other mechanisms such as NER, homologous recombination and fanconi anemia pathways involve in the DPC repair with/without the proteolytic processing (11, 19, 20). Recent discovery of DNA-dependent mammalian protease, SPRTN (SprT-like N-terminal domain) is required to proteolyze DPC *in vivo*. It was observed that the function of SPRTN was compromised in Ruijs-Aalfs syndrome patients suffering from accelerated aging and increased incidence of liver cancer (21).

Epigenetics is the term used to define the interaction of the genome and the environment, which influences the gene expression in higher organisms (22). Epigenetic modifications are inheritable, occur as a result of DNA methylation, histone modification and nucleosome positioning (23). These are important in the cellular mechanisms including micro-RNA expression, DNA protein

cross-link formation, suppression of transposable element mobility, cellular differentiation, embryogenesis, X-chromosome inactivation and genomic imprinting (23). They also play an important role in the cancer initiation and progression. One of the common bases which undergo methylation in humans is deoxycytosine (dC) to form 5-methylcytosine (5mC), catalyzed by DNMT family of enzymes (23, 24). 5mC undergo oxidation to form 5-hydroxymethylcytosine (5hmC), which on further oxidation in a stepwise reaction form 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) by the action of TET (ten-eleven translocation) family dioxygenases (24) as shown in scheme 3.1. Low levels of 5fC bases have been detected in all mammalian tissues (25-27). It was recently discovered that 5-formylcytosine (5fC) forms reversible Schiff base conjugates with histone proteins; these can be reductively stabilized to form stable amino conjugates (28, 29). 5fC forms reversible Schiff base cross-links with histone proteins both *in vitro* and in human cells to form DNA protein cross-links (DPC), which influences the gene expression levels in humans (28, 29). These DPCs are acted upon by the proteolytic enzymes which result DNA peptide cross-links (DpCs). In this research, we studied the mutagenicity and toxicity induced by adduct, 5fC-DpC and its TLS bypass in human cells.



SCHEME 3.1: Methylation and oxidation of cytosine by DNMT transferases and TET-family dioxygenases respectively to form 5-formylcytosine.

In comparison, relatively little is known about the interactions of DPCs with DNA replication machinery. Although in the early 1990s DPC formation and mutagenesis in mammalian cells were shown (30), the majority of the reports published subsequently either failed to link formation of DPCs with mutations (31, 32) or suggested that DPCs may not induce mutagenesis in mammalian cells (32). More recent research using advanced tools, however, established that DPCs containing large proteins completely block replicative bypass but that peptide containing DpCs allow TLS (11, 33-39).

Our previous studies with a model DpC have established a key role of TLS polymerases, such as hpol **κ** and **η**, in catalyzing DNA replication past DpC lesions (37). Unlike replicative polymerases, whose tight active sites undergo conformational changes upon binding the correct dNTP, TLS polymerases of the Y family have more open sites that enable them to bypass bulky DNA lesions (40, 41). We found that 5fC conjugated to histones H2A or H4 completely inhibited

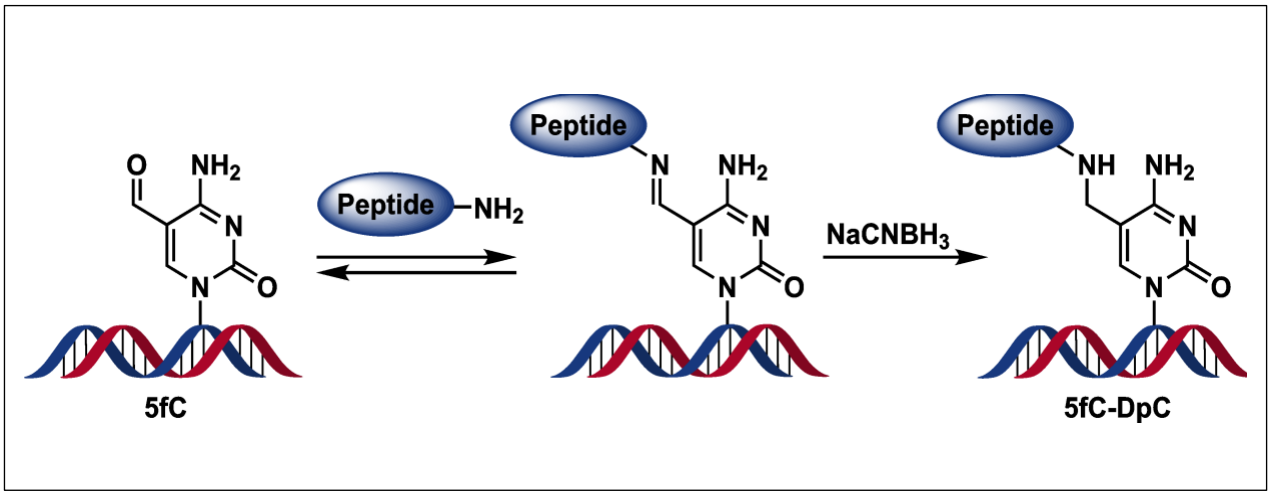
DNA replication *in vitro* but that TLS by Y-family polymerases occurred when the proteins were subjected to proteolytic digestion (38). 5fC-mediated cross-links to 11-mer or 31-mer peptides were bypassed by hpol **η** and **κ** in an error-prone manner, including targeted C->T transitions and (-1) frameshift deletions. Similar mutations also occurred when a plasmid containing the 11-mer DpC is replicated in HEK 293T cells (38).

The main purpose of this work was to establish the roles of the TLS DNA polymerases in both error-free and error-prone bypass of DpCs in living cells. We examined replication of a plasmid containing a site-specific, structurally defined cross-link between 5fC and an 11-mer peptide in HEK 293T cells as well as in the cells subjected to knockout and/or knockdown of one or more TLS polymerase(s). To focus on replication rather than repair, we employed a single-stranded plasmid with DpC, as DNA damages in single-stranded DNA are refractory to repair by most common types of DNA repair pathways. We have also investigated the ability of hpol **δ** and **ε** to bypass the DpC lesion *in vitro*.

3.2 SYNTHESIS OF DpC:

A 23-mer oligodeoxynucleotide (5'-AGG GTT TTC CXA GTC ACG ACG TT-3'), where X represents 5fC covalently bonded to an 11-mer peptide at lysine (K), RPKPQQFFGLM-$CONH_2$, was synthesized and characterized as described in the literature (28). The procedure for preparation was outlined briefly in scheme 3.2, and its characterization and purity were performed by MS. The aldehyde group of 5fC forms a reversible Schiff base with the side chain of the third amino acid, lysine, of the polypeptide, which was stabilized by $NaCNBH_3$ reduction. The oligonucleotide-polypeptide conjugate was purified by electrophoresis on a 20 % polyacrylamide gel containing 7

M urea (denaturing PAGE), followed by Sep-Pac C18 SPE desalting and characterized by mass spectrometry.



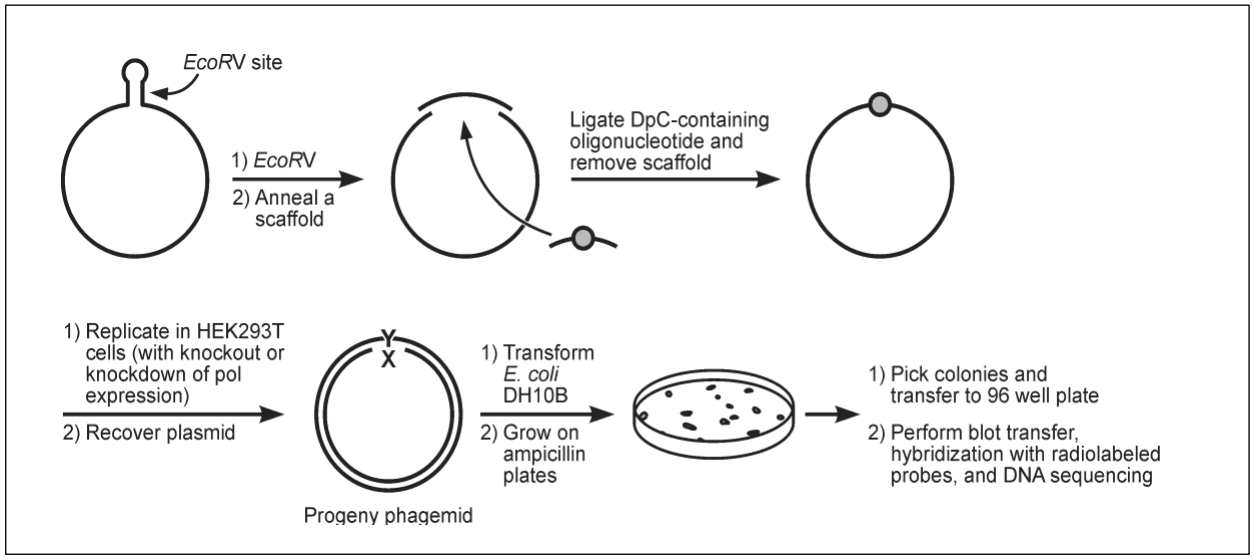
SCHEME 3.2: Preparation of 5fC-conjugated DNA peptide cross-link by forming Schiff base with 5fC and Lysine of the peptide, further stabilized by reduction.

3.3 RESULTS:

3.3.1 Replication of DNA peptide cross-link in human cells:

The DpC-containing 23-mer oligonucleotide (*5fC-DpC* in Scheme 3.2) was ligated into a gapped pMS2 plasmid for 18 h at 16 ºC, followed by 14 h at 4 ºC, to generate a recombinant plasmid vector. Prior to the ligation, the 5fC-DpC was phosphorylated twice for 1 h each time at 37 ºC to increase the efficiency of the reaction. The DpC construct and an unmodified plasmid (containing a different sequence at the ligation site) were co-transfected into HEK 293T cells. The unmodified vector served as an internal control of transfection efficiency. Following 24 h incubation, where cells completed one round of replication, plasmid DNA was isolated and used to transform in

E.coli DH10B cells (Scheme 3.3). The resultant colonies were analyzed by oligonucleotide hybridization followed by DNA sequencing.



SCHEME 3.3: Recombinant pMS2 vector construction with 23-mer DNA peptide cross-link adduct followed by mutational screening using Southern blot hybridization and confirmed by DNA sequencing.

3.3.2 Complete mutation profile induced by DpC and role of TLS polymerases:

The figure represents the complete mutation profile of DpC in HEK 293T cells and in various polymerase (pol)-deficient cells. The mutations obtained were categorized into targeted and semi-targeted mutations as shown in figure 3.1. Targeted represents the base substitutions/deletions at the lesion-bearing site. Semi-targeted represents the base substitutions/ deletions at the 5' and 3' flanking regions of targeted site. Nearly 14 % of plasmid progeny recovered from HEK 293T cells, transfected with the DpC-vector were mutants, which included 9 % targeted and 5 % semi-targeted base substitutions and frameshift mutations (deletions). Knockout/knockdown (KO/KD) of TLS polymerases resulted in reduced mutation frequency (MF) upon replication past DpCs, which was most pronounced upon knockdown of either hPol **ι** or hPol **ζ**. Targeted mutations were reduced by

50 %-80 %, from 8.6 ±1.9 % in HEK 293T cells to 2.9 ± 0.4 % in pol **ι** KD cells. Similarly, it was reduced to 3.2 ± 0.6 % in pol **ζ** KD cells, a reduction of 45 %-75 %. Then, a double knockdown of pol **ι/ζ** was performed and the MF was not further reduced. Various pol-deficient cells were generated by siRNA knockdown and CRISPR-Cas9 techniques, in which DpC-vector was replicated. Among these double polymerase KO/KD experiments, pol **ι/ζ** KD and pol **η/κ** KO show similar reduction in the MF. Further, replication of DpC-vector in triple polymerase KO/KD cells was performed and the maximum reduction in MF was observed when the cells are devoid of polymerases **η/κ/ζ**. Here in pol **η/κ/ζ** KO/KD cells, the MF was reduced from 13.5 ± 1.7 % in HEK 293T to 4.8 ± 0.7 %.

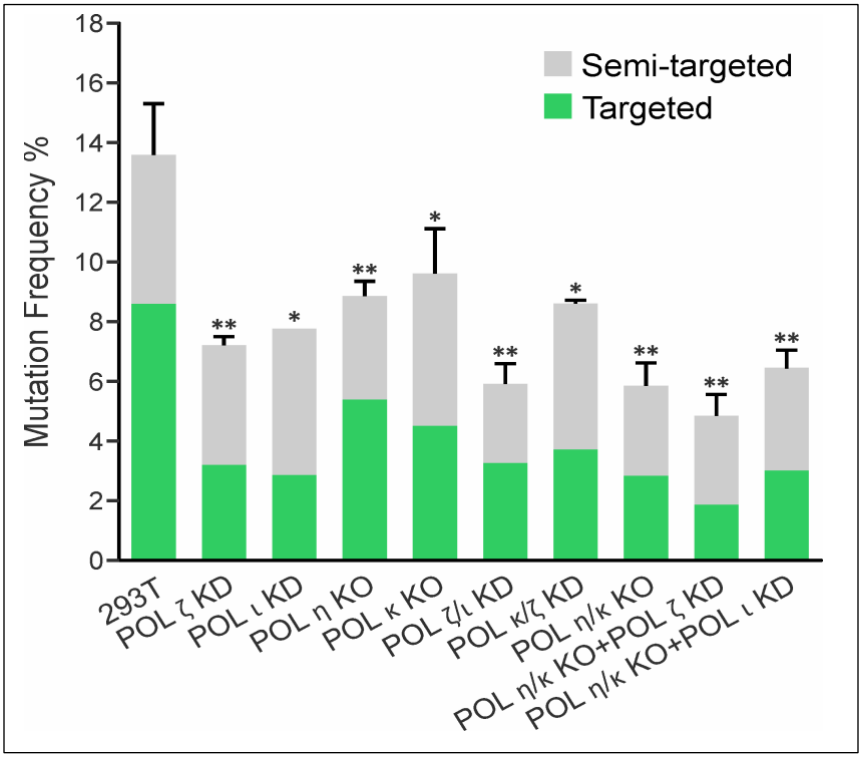


FIGURE 3.1: Targeted and semi-targeted mutations induced in the progeny from the 5fC–linked DpC construct in HEK 293T (293T) and various polymerase KO or KD cells (as indicated). The data represent the mean and standard deviation (of total MF) from two to five independent experiments. The statistical significance of the difference in MFs between HEK 293T and TLS pol knockouts and/or knockdowns (KD) was calculated using two-tailed, unpaired Student's t-test (*p < 0.05; **p < 0.005).

Of the four types of targeted mutations, the prevalent ones found in all the experiments were C->T and C-deletion as shown in figure 3.2. Other infrequent mutations were C->G, C->A. In 293T cells, targeted mutations were comprised of 6.7 % C->T transitions, 0.6 % C->G transversions, and 1.3 % targeted C deletions. Similarly, C->T mutations were reduced by 40 %-80 % and 30 %-70 % upon single knockdown of hPol **ι** and hPol **ζ** respectively. With reference to the total MF data, C->T mutations were reduced to 1.4 ± 0.2 % in triple KD/KO of pol **η**/**κ**/**ζ** cells from 5.9 ± 1.4 % in HEK 293T cells.

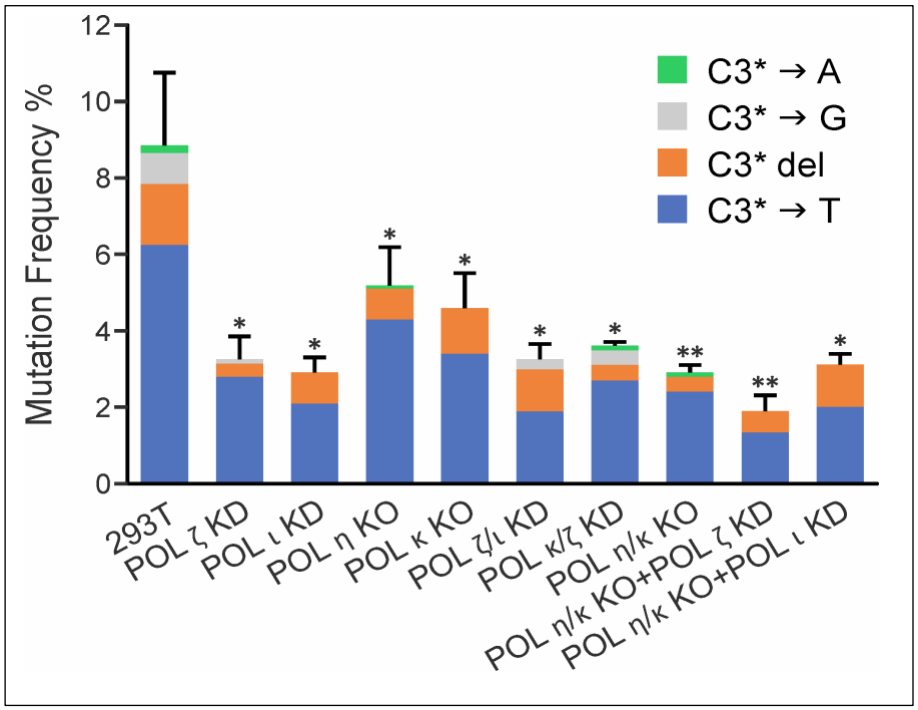


FIGURE 3.2: The types and frequencies of targeted mutations induced in the progeny from the 5fC-linked DpC construct in HEK 293T cells and various polymerase KO or KD cells (as indicated). The data represent the mean and standard deviation (of the total targeted MF) from two to five independent experiments. The statistical significance of the difference in targeted MFs between HEK 293T and TLS pol knockouts (KO) and/or knockdowns (KD) was calculated using two-tailed, unpaired Student's t test (*p <0.05; **p < 0.005).

Semi - targeted base substitutions and 1 - 6 base deletions around the DpC site comprised ~33 % of total mutations in HEK 293T cells. Nearly half of the semi-targeted mutations were 1 - 6 base deletions, whereas insertion mutations did not occur. Of the various semi-targeted base substitutions, C–>T at several different cytosine sites in the adduct were predominant. Upon knockout and knockdown of TLS polymerases, the frequency of semi-targeted mutations decreased in some cases, but no clear pattern emerged, and the changes were not statistically significantly different. In some knockout/knockdown experiments, the percentage of semi-targeted mutations increased to more than 50 % of the total mutations. Taken together, the results from TLS polymerase knockout/knockdown experiments are indicative of an involvement of TLS polymerases hPol **ι**, hPol **η**, hPol **κ**, and hPol **ζ** in error-prone bypass of 5fC-mediated DpC lesions in human cells.

3.3.3 TLS efficiency:

Figure 3.3 represents the bypass efficiency of DpC in HEK 293T cells, along with various experiments of single/double and triple knockout/knockdown of TLS polymerases (pol **η**, pol **ζ**, pol **κ**, pol **ι**). TLS efficiency was determined as the percentages of the colonies recovered from the lesion-containing plasmid relative to the internal control. In HEK 293T cells, the replication efficiency of the DpC-containing plasmid was 98.5 ± 1% compared with an unmodified control plasmid (set as 100 %).We next examined the effects of 5fC-DpCs on DNA replication in various TLS polymerases-deficient cells (hPol **η**, hPol **ζ**, hPol **κ**, and hPol **ι**). Surprisingly, the TLS efficiency did not change upon knockout of hPol **η** and hPol **κ** or knockdown of hPol **ι** and hPol **ζ**. TLS remained above 93 % even in double and triple knockout/knockdown of TLS polymerases.

This indicates that TLS polymerases are not required for bypass of 5fC-DpCs in human cells and that it can be bypassed by replicative DNA polymerases.

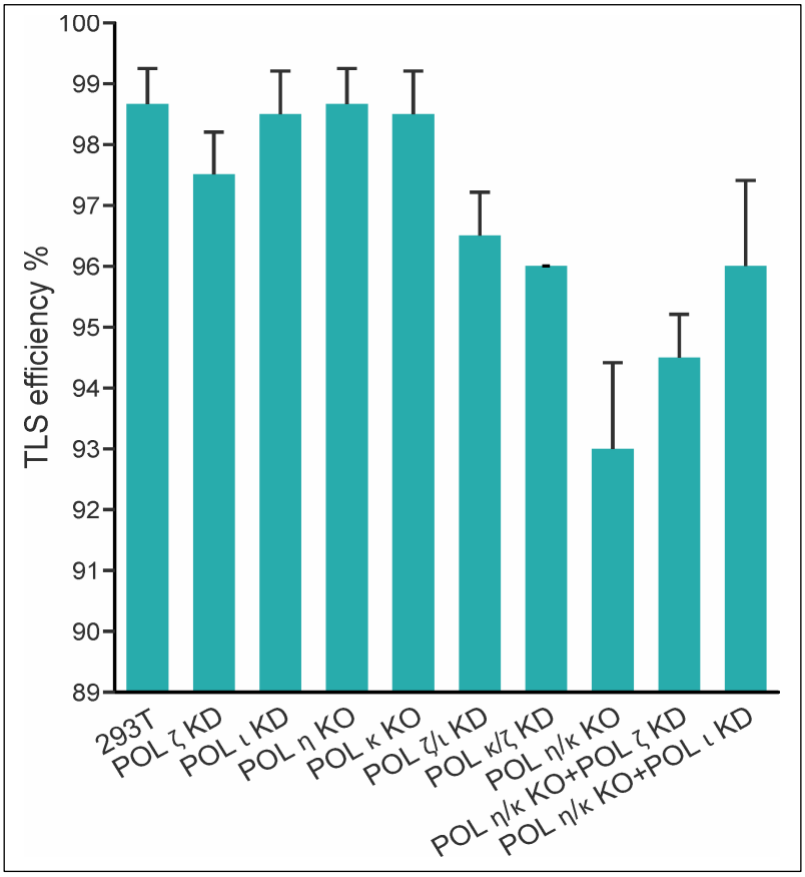


***FIGURE 3.3**: The extent of replicative bypass (or TLS efficiency) of 5fC-linked DpC in HEK 293T cells with or without various TLS polymerases. Percent TLS of the DpC construct in different polymerase knockout or knockdowns was measured relative to an internal control DNA. The data represent the mean and standard deviation of results from three independent experiments. HEK 293T cells were treated with negative control siRNA (293T), whereas the other single or double polymerase knockouts (KO) or knockdowns (KD) are indicated below on the x-axis.*

3.3.4 *In vitro* replication of a DpC lesion by replicative DNA polymerases δ and ε using primer extension assay:

Our observation of efficient replicative bypass of the 5fC-linked DpC lesion in HEK 293T cells even with radically reduced levels of TLS polymerases suggests that these specialized polymerases are not required for DpC lesion bypass in human cells. Therefore, we investigated the possibility that this bulky adduct can be bypassed by replicative DNA polymerases **δ** and **ε**. Standing-start *in vitro* primer extension assays were conducted in the presence of hPol **ε** (left panel) and hPol **δ** (right panel) as represented in the figure 3.4. Primer–template complexes were created by annealing ^{32}P-labeled 12-mer primers to the 23-mer template containing 5fC control or the 11-mer peptide DpC. *In vitro* replication was initiated by the addition of the corresponding DNA polymerase and a mixture of dNTPs. Primer extension reactions were quenched at selected time points prior to loading onto 20 % denaturing polyacrylamide gel.

As shown in figure 3.4, recombinant hPol **ε** (left panel) was able to extend the primer past unconjugated 5fC to form a full-length 23-mer product, although a significant fraction of the primer remained unchanged, even after a 30-min reaction. Primer extension using the DpC template was even less efficient, but a small part of the full-length product was formed in 5 min, which gradually increased over a 30-min time period. In comparison, hPol **δ**-catalyzed replication of the control template was significantly more efficient, with more than 95 % of the primer extended to the full-length product in 5 min (right panel). Primer extension using the DpC-containing template was much slower, but the band corresponding to the full-length product continued to increase during the 30 min incubation. This suggests that both hPol **ε** and hPol **δ** can bypass the 5fC-conjugated DpC, albeit more slowly than the 5fC control. In addition, both

replicative polymerases exhibited exonuclease activity, so a significant portion of the primer were excised at longer incubation times.

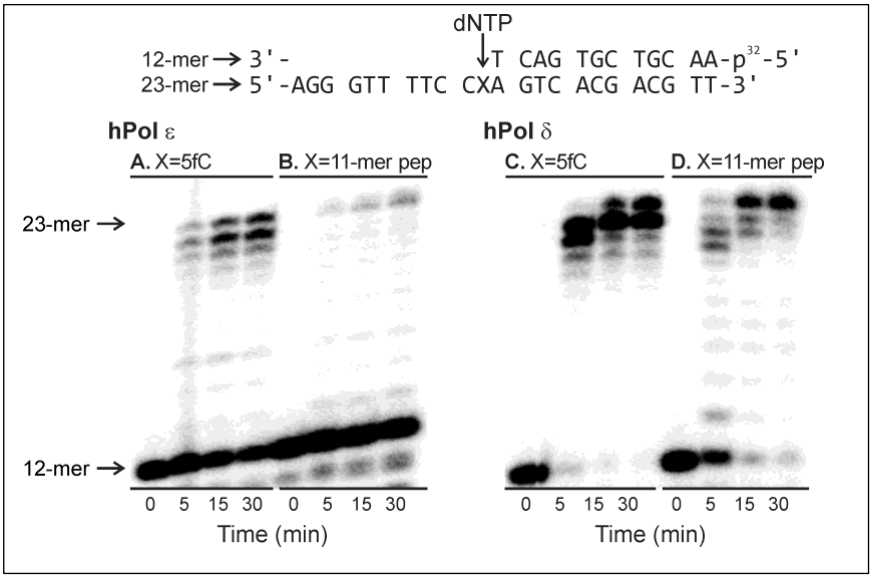


FIGURE 3.4: Primer extension assays for replication bypass of 5fC-mediated DNA peptide cross-links by hpol ε and hpol δ (A-D), the ^{32}P-labeled 12-mer primer was annealed with a 23-mer template containing 5-formyl-dC (A and C) or the 11-mer peptide cross-link RPKPQQFFGLM (B and D). Polymerase reactions were initiated by addition of DNA polymerases and a mixture of dNTPs and quenched at selected time points prior to loading onto 20 % denaturing PAGE.

3.3.5 Fidelity of replication of a DpC lesion by replicative DNA polymerases ε and δ by nucleotide incorporation assay *in vitro*:

Figure 3.5 & 3.6 represent the single nucleotide insertion experiments conducted, to investigate the fidelity of hPol **ε** and hPol **δ** respectively upon *in vitro* replication of the DpC-containing template. For hPol **ε**, the correct nucleotide (dGTP) was preferentially incorporated opposite both DpC and the 5fC control. Gel electrophoresis analyses revealed that three successive dGs were incorporated because of the 3'-XCC sequence of the template strand.

Although low levels of dTTP and dATP misincorporation were noted opposite 5fC, no noticeable misincorporation opposite DpC was detected. Similar results were obtained for hPol **δ**- dGTP was the only nucleotide inserted opposite DpC, whereas dGTP and low levels of dATP were incorporated opposite the 5fC control. To determine the catalytic efficiency of dGTP incorporation by hPol **δ** opposite DpC and 5fC, steady-state kinetic experiments were conducted. The same primer–substrate complexes were used, and the primer extension reactions were conducted in the presence of hPol **δ** with increasing concentrations of dGTP (5-500 μM) as shown in the figure. To ensure steady-state conditions, a 9-90 molar excess of DNA over hPol **δ** was employed. Steady-state kinetic parameters were determined from Michaelis-Menten curves (Table 3.1). k_{cat}/K_m values for dGTP incorporation opposite 5fC and DpC were calculated as 0.056 and 0.008 μM^{-1} min^{-1}, respectively, indicating that the presence of the peptide lesion significantly inhibits DNA polymerase activity. Overall, these results revealed the ability of replicative DNA polymerases to catalyze error-free nucleotide addition opposite 5fC derived DpC lesions. Perhaps this is not surprising, as such lesions routinely form at epigenetic marks in human cells and, thus must be tolerated by the cellular machinery.

Polymerase	Template	Incoming nucleotide	K_{cat} (min^{-1})	K_m (μM)	K_{cat}/K_m (μM^{-1} min^{-1})
hPol **δ**	5fC	dGTP	1.30 ± 0.08	23 ± 5	0.056 ± 0.013
	11 - mer peptide	dGTP	0.13 ± 0.007	17 ± 4	0.008 ± 0.002

TABLE 3.1: Steady-state kinetics parameters for single-nucleotide incorporation opposite 5fC or the 5fC-11-mer peptide (RPKPQQFFGLM) conjugated to the C5 position of cytosine by hpol δ.

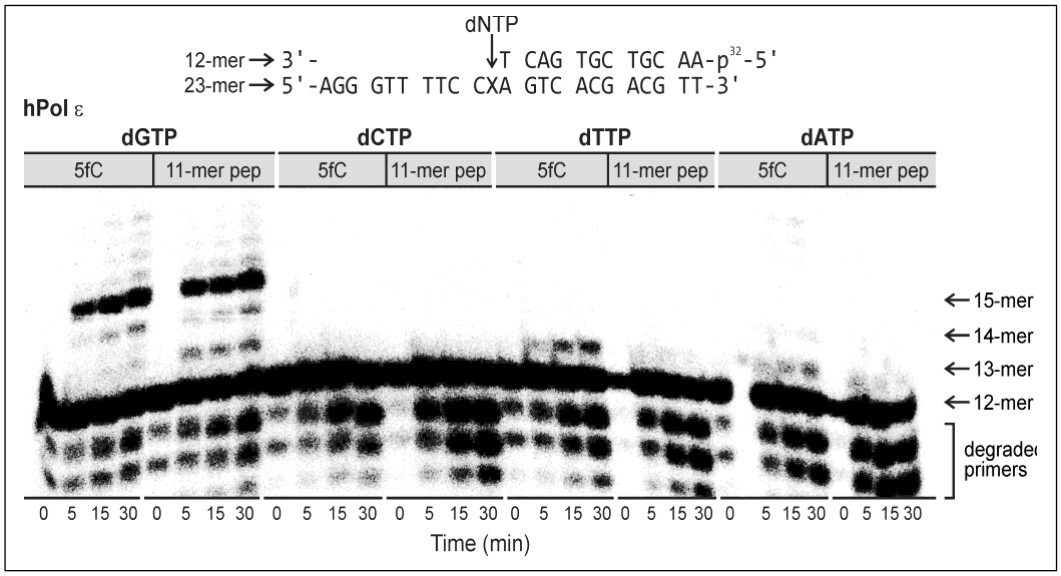


FIGURE 3.5: Single-nucleotide insertion opposite 5fC (control) and the 5fC-11-mer peptide cross-links by hpol ε. Template-primer complexes were incubated with hpol ε in the presence of individual dNTP and then quenched at preselected time points. X denotes 5 -formylcytosine conjugate to RPKPQQFFGLM.

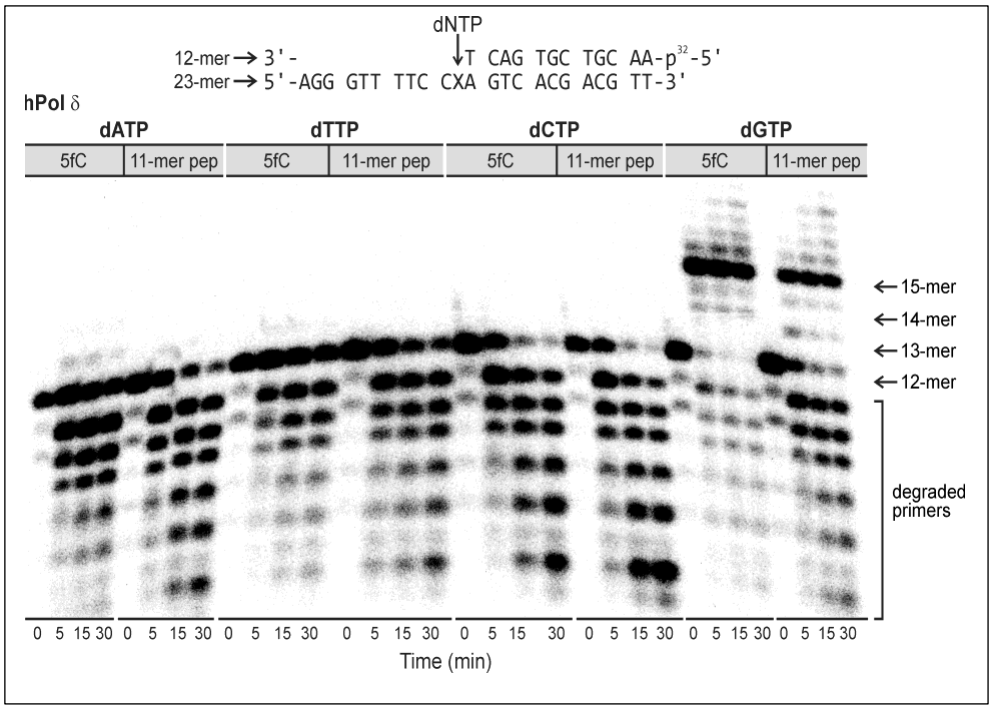


FIGURE 3.6: Single-nucleotide insertion opposite 5fC (control) and the 5fC-11mer peptide cross - link by hpol δ. Template-primer complexes were incubated with hpol δ in the presence of individual dNTP and then quenched at pre-selected time points. X denotes 5-formylcytosine conjugate to RPKPQQFFGLM.

3.4 DISCUSSION:

DPC formation in cells was first reported as early as in the 1960s (3). In the early 1990s, it was shown that vanadate (VO_3^-) produces DPCs and causes mutations in mammalian cells (30). Most other reports in the latter part of the 20th century, however, either failed to associate formation of DPCs with mutations (31, 32) or indicated that DPCs cause cytotoxicity and clastogenicity but may not induce mutagenesis in mammalian cells (42). Research in the last two decades, in contrast, unambiguously established that DPCs containing large proteins pose strong blocks of DNA replication, whereas certain peptide-containing DpCs allow error-prone replicative bypass both *in vitro* and *in vivo (11, 33-38)*. For example, Lloyd and coworkers (34, 43) have shown that DpCs covalently linked to the N^2 position of dG blocked DNA replication, whereas the same peptide conjugated to the N^6 position of dA was bypassed and induced small number of mutations in both *E. coli* and simian kidney cells. *In vitro* studies showed that *E. coli* pol II, pol III, and pol V cannot replicate past these DpCs but that pol IV is reasonably proficient in their bypass (34). Moreover, Pol **κ** is highly efficient and accurate in TLS of the dG-N^2-DpCs, as it is with other dG-N^2 adducts (34). Indeed, the types and frequencies of DPC - induced mutations appear to be strongly dependent on the site and structure of the cross-links as well as the types of cells in which they are replicated. We have shown that site-specifically modified templates containing 10 to 20-mer peptides conjugated to uracil, 7-deazaguanine, or 5fC, unlike full-length protein DPCs, are subject to bypass by Y-family DNA polymerases. Although proteins and large peptides (>20 amino acids) conjugated to the C5 position of dT completely blocked Pol **η** and **κ**, smaller C5-dT DpC

conjugates (<10-mer peptides) were bypassed in an error-prone manner, giving rise to large numbers of deletions and point mutations (35, 44).

Depletion of one or more TLS DNA polymerases has become an attractive approach to determine their role in lesion bypass, which we employed in this study. A limitation, however, was that we were able to generate only a limited number of knockout cell lines and used the siRNA knockdown approach for the rest. A strict comparative quantitative analysis, therefore, is unwarranted. Even so, the critical role of TLS polymerases in the mutagenic outcome was evident.

In one of our earlier reports, TLS efficiency of a model DpC containing a 10-mer Myc peptide covalently linked to C7 of 7-deaza-dG was 76 % in HEK 293T cells compared with 100% progeny derived from the control DNA, suggesting that this lesion slowed down DNA replication (37). In contrast, DpCs conjugated to the C5 position of 5fC examined in this study, allowed a TLS efficiency comparable with that of the 5fC control in HEK 293T cells. Additionally, TLS efficiency was barely altered in cells with or without TLS polymerases, suggesting that none of these polymerases were essential for TLS of the C5-5fC-conjugated DpC lesion. This led us to hypothesize that C5-5fC DpC-containing DNA may be bypassed by replicative DNA polymerases. Indeed, *in vitro* replication experiments with recombinant hPol **δ** and hPol **ε** revealed that both polymerases can catalyze DNA synthesis past the 5fC-linked 11-mer peptide lesion, albeit more slowly than 5fC. Remarkably, nucleotide incorporation opposite the DpC by hPol **δ** and hPol **ε** was accurate, and no nucleotide misincorporation opposite the lesion was detected. Overall, our results indicate that hPol **δ** and hPol **ε** can bypass the C5-5fC-conjugated DpC in an error-free manner. However, because of the low efficiency of bypass, TLS polymerases are likely to be recruited to the replication fork, leading to nucleotide misincorporations. The mechanism of

nucleotide misincorporation can be rationalized from our recent molecular dynamics' simulations of the hPol **η**-DNA ternary complexes, which revealed that the 11-mer peptide can be accommodated in the DNA major groove side. It also showed that the adducted 5fC forms a stable pair with the incoming dATP via wobble base-pairing in the polymerase active site, thereby causing C->T transitions (38). The targeted C deletion, on the other hand, can be explained by a slippage mechanism, when the incoming dG opposite the adducted 5fC realigns to the 5'-adjacent C, thereby causing the one-base targeted deletion (38). The cellular mutagenesis results for 5fC -induced DpCs differ from our earlier findings for DpCs conjugated to the C7 of 7-deaza-dG (37). In that study, replication of DpC-containing plasmids produced both targeted (20 % targeted G-> A and G->T) and semi-targeted (15%) mutations in HEK 293T cells (37). TLS efficiency and targeted mutations were reduced upon siRNA knockdown of pol **η**, pol **κ**, or pol **ζ**, indicating that they participated in error-prone bypass of 7-deaza-dG-linked DpC lesions (37). However, the semi-targeted mutations at G_5 position were only reduced upon knockdown of pol **ζ**, suggesting its critical role in this type of mutations (37). Even though targeted and semi-targeted mutations were observed in both studies, each structurally distinct DpC exhibited a unique spectrum of mutations. We postulate that the semi-targeted mutations induced by DpCs arise from local destabilization of the DNA helix in the presence of bulky DpC lesions and from interference of nucleotide incorporations near the lesion site by the long arm of the peptide, which can adopt many different conformations. Future structural studies will likely shed light on the validity of this hypothesis and the mechanistic details of semi-targeted mutagenesis by the DpCs.

Although this study was conducted with the 5fC-DpC situated in single stranded DNA, which is not repaired by most DNA repair systems, immediately after TLS, the DpC-containing plasmid may become a substrate for DNA repair. 5fC itself is repaired by base excision repair (45), whereas

the 5fC-DpC conjugate is likely to be an NER substrate (12, 18, 19). In this study, we did not attempt to determine the role of DNA repair in mutagenesis, but, in the future, it would be of interest to determine the interplay of DNA repair with mutagenesis induced by this DpC.

3.5 CONCLUSION:

In conclusion, our results indicate that polymerase bypass of 5fC-conjugated DpC lesions by TLS polymerases gives rise to mutations in human cells. Even for such bulky DNA adducts, the specialized bypass polymerases of the Y and B families are not required for bypass of the 5fC-polypeptide cross-link. Replicative hPol **δ** and hPol **ε** could bypass the 5fC-linked 11-mer peptide lesion in an error-free manner. However, because of the low efficiency of DpC bypass by hPol **ε** and hPol **δ**, TLS polymerases are recruited to the replication fork, leading to nucleotide misincorporations and mutagenesis in human cells.

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CHAPTER – 4

Mutagenic Effects of a 2-Deoxyribonolactone -Thymine Glycol Tandem DNA Lesion in Human Cells

4.1 INTRODUCTION:

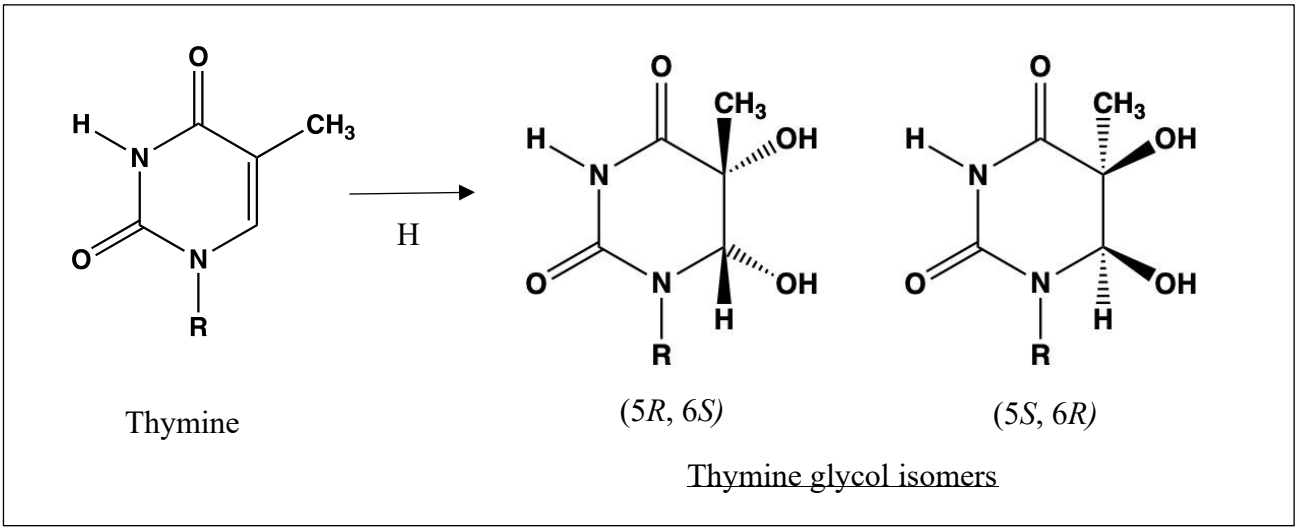
Reactive oxygen species (ROS) (1,2) are produced by various endogenous and exogenous agents. The hydroxyl radical (OH•), one of the highest generated radicals (1,3), interact with the DNA nucleobases to form a peroxyl radical (O-O•) at C5 position (4). Majority of the peroxyl radicals under aerobic conditions, react with the 5' or 3' adjacent nucleobases to form tandem lesions (4,5). Tandem lesions are contiguously damaged nucleotides present adjacent to each other in the DNA. These form an important part of the clustered DNA damages (5-7).

In this chapter, the mutagenicity and cytotoxicity of tandem lesion (5'-LTg-3') comprising of 2-deoxyribonolactone (L) & thymine glycol (Tg) was studied. Along with a compare and contrast study among 5'-LTg-3' and isolated lesions L and Tg, when replicated in human cells. Due to their close proximity of the DNA damages, the tandem lesions are more resistant to repair than their individual presence in the DNA (8).

4.1.1 Formation of thymine glycol (Tg):

Thymine glycol (Tg) is the most common oxidation product of thymine when exposed to ionizing radiation (9-11). OH• radicals react with thymine at C5 and C6 position, forms two predominant isomers (*5R, 6S*) and (*5S, 6R*) (12,13) as shown in scheme 4.1. It is reported that the *5R* and *5S* isomers were formed in equal proportions as a result of **γ** - radiolysis, but oxidation of thymine results in the preferential *5R* isomer (11,14). Hence, the replication bypass of *5R* isomer was studied here. Upon the addition of hydroxyl groups at C5 and C6 positions, thymine loses its aromaticity to form a non-planar structure (11). Tg lesions in humans can be repaired by long patch BER (15,16), by the action of DNA glycosylase/AP lyase NTH1 (17) and to a small extent by

NER (16,18). Since all the Tg lesions cannot be repaired, they proceed to replication bypass which is carried out by TLS.



***SCHEME 4.1*: Oxidation of thymine to form thymine glycol (Tg) isomers (5R, 6S) and (5S, 6R).**

4.1.2 Formation of 2-deoxyribonolactone (L):

2-deoxyribonolactone (L) is the most common oxidized abasic sites found in the DNA (19-21). It is formed by the oxidation of C1' of an abasic site (19,20). C1' site is buried in the minor groove of the DNA rendering it less accessible for the oxidation to occur, hence the frequency of the formation of single independent lesion, L was reported to be very less (22,23). However, it is reported that the formation of L, as 5'-component of a tandem lesion is more frequent as the peroxyl radical on the 3' pyrimidine base component facilitates it (24-26). Formation of these peroxyl nucleobase radicals are more common, when exposed to **γ**-radiolysis (26). Previous kinetics studies reported that the peroxyl radical mediated formation of L is favored 4-5 fold than the direct formation of L (27). L is naturally formed when DNA is exposed to anti-tumor agents such as

NCS, because NCS binds to the minor groove of DNA and thereby overcomes the C1' inaccessibility (21,24,28).

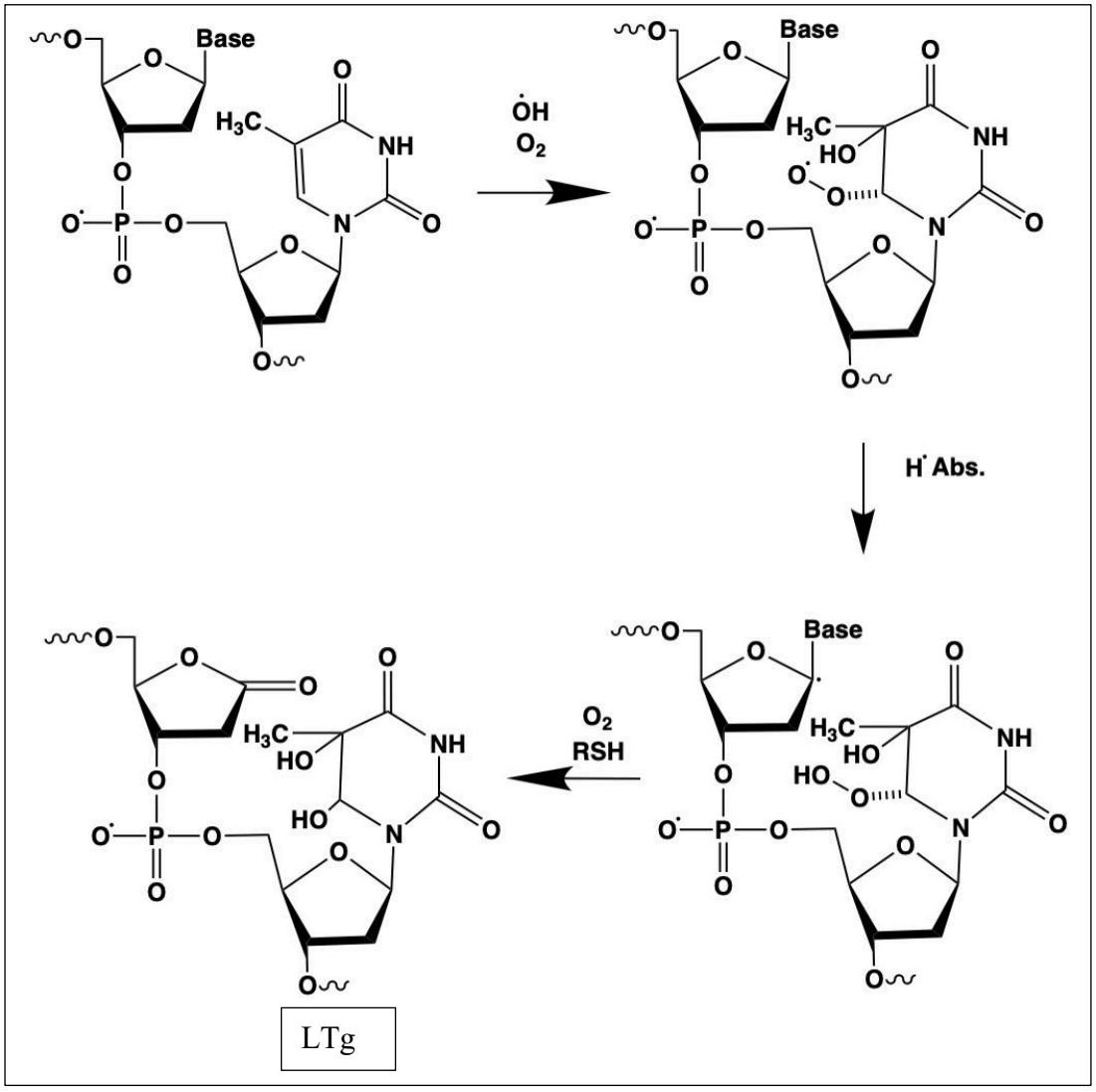
L is formed by either of the two processes proposed, which are two-electron and one-electron oxidations (29,30). Two-electron oxidation includes an intermediate nucleobase radical formation which proceeds to further oxidation to form L (31-33). The latter process begins with the direct H-atom abstraction from the nucleobase (29,30). L is repaired by long-patch BER process, which is proceeded through strand displacement by polymerase **β** and excision by FEN1 enzyme (15,34).

4.1.3 Formation of tandem lesion (LTg):

Tandem lesions are regarded as a major class of products formed from the pyrimidine nucleobase radicals, as a result of ionizing radiation (24-26). It should be noted that about 82 % of peroxyl radicals produced in this manner, will attack either 5' or 3' nucleotides to form tandem lesions, which depends on the radical bearing nucleobase (26,27,35-37). In this case, the peroxyl radical attached to thymidine can only abstract C1'-H atom from 5'-nucleotide. About 11 % of tandem lesions in the cells contains 2-deoxyribonolactone as the component with Tg (27). Tandem lesion (LTg) as a whole, is responsible for specific biochemical effects in the BER repair process in the cells. L-component forms DPCs irreversibly with the BER proteins, thereby inhibits their function (15,38). Particularly, the enzymes endonuclease III and DNA polymerase **β** are crosslinked to DNA by the Schiff base formation with their lysine side chains (15,39). It also results in the increased formation of DSBs (40). Both isolated L and tandem LTg are alkali labile lesions which cleave when treated with NaOH (0.1 M, 37 ºC, 20 min) (41-43).

The postulated mechanism (5,26-28) of formation of LTg is shown in scheme 4.2 and described below. When exposed to ROS, the $OH^{\bullet}$ radical breaks the double bond, forms C5 - radical. Under

aerobic conditions, C5 radical transforms into a peroxyl radical. This peroxyl radical will further abstract the C1'-H atom from 5'-adjacent nucleotide to form 2-deoxyribonolactone (L), and dT into thymine glycol (Tg), thus results in tandem lesion (LTg).



SCHEME 4.2: Formation of tandem lesion (LTg), an oxidized abasic site resulted from the attack of OH• radical on thymine, under aerobic conditions.

4.2 SYNTHESIS STRATEGIES:

4.2.1 Synthesis of oligonucleotides containing Tg (44):

To understand the implications in human cells, organic synthesis of these lesions was performed and were site specifically incorporated in the oligonucleotides. All the oligonucleotides used in this study, were of 16-mer length, synthesized on an Applied Biosystems Inc. 394 DNA synthesizer. The (*5R,6S*)-thymine glycol phosphoramidite was purchased from Glen Research Inc. and was deprotected as described in literature (5). The solution was treated with 3 M NH_4OAc and EtOH for precipitation of modified oligo. The Tg - containing oligo was further purified by 20 % PAGE gel electrophoresis.

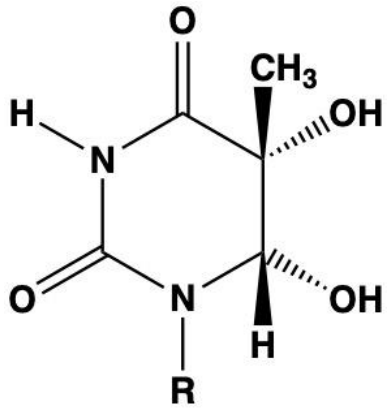
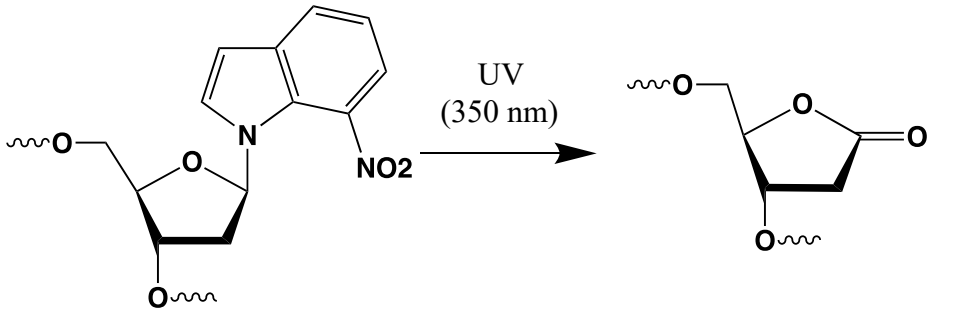


FIGURE 4.1: (5R, 6S) isomer of Tg-adduct in the sequence 5'- GAAGACCTTgGGCGTCC -3'

4.2.2 Synthesis of oligonucleotides containing L and LTg:

Since L and LTg are alkali-labile lesions, the C1' site was protected by chemical groups which in turn serve as photochemical precursors (45-47). These precursors were site-specifically incorporated into a 16-mer oligonucleotide, which when exposed to UV radiation at 350 nm form L and LTg and were immediately used for the mutagenesis assay. The photochemical precursor specific for LTg is 7-nitroindole nucleoside, with adjacent 3'-Tg (45). The photochemical precursor for L is a model of Norrish I type precursor (48,49). The structures of precursors were shown in the scheme 4.3. After the synthesis of precursor containing oligonucleotides, they were treated with 3 M NH_4OAc and EtOH for purification. Purity of these oligos was checked by 20 % PAGE electrophoresis. The purified oligonucleotides were characterized by ESI-MS and their molecular weights were calculated (24).



***SCHEME 4.3*: Formation of 2-deoxyribonolactone (L) from photochemical precursor, exposed to UV radiation at 350 nm. The precursor was incorporated in the oligonucleotide sequences shown.**

5'-GAAGACCTL*GGCGTCC-3'
5'-GAAGACCL*TgGGCGTCC-3'

4.2.3 Modified pMS2 vector preparation with site specific incorporation of Tg, L, LTg:

Single stranded pMS2 plasmid extracted form *E.coli*, was employed to insert the 16-mer adducts containing, Tg, L, and LTg. pMS2 vector contains a restriction site which was digested by *EcoR*V enzyme. A 62-mer scaffold was then annealed to the gapped plasmid. The adducts with Tg, LpTg and Lp (p-photochemical precursor for L) were phosphorylated at 5' end using T4 polynucleotide kinase. The phosphorylated adducts were then rested on ice for 10 min. To the vials with Lp, LpTg, add 0.1 M HEPES buffer (pH 7, final concentration-0.01 M) and exposed to the UV radiation at 350 nm using a photochemical reactor (Rayonnet) for 20 min at 4 ºC. After 20 min, the vials were further cooled on ice for 10 min. To these respective phosphorylated adducts, the pMS2-annealed scaffolds were mixed and incubated for ligation at 16 ºC for 2 h using T4 DNA ligase enzyme to obtain re-circularized modified vector. Later, the scaffold was removed by T4 DNA polymerase by incubating at 16 ºC for 1 h. Immediately after this step, the unreacted oligos, salts and enzymes were removed by using Amicon®-100 k MWCO ultracentrifugation columns, which retain the modified vector. These were quantified by 1.1 % agarose gel electrophoresis.

4.2.4 Replication of isolated-Tg, isolated-L and tandem-LTg:

Modified pMS2 vectors with site specifically incorporated Tg, L and LTg were transfected in HEK 293T cells by transfection using lipofectamine 2000 and opti-MEM reagents. The cells were then harvested, DNA was extracted, and the mutants were screened by Southern blot hybridization as described in the experimental section. The mutants selected were sent for commercial sequencing analysis to obtain the position and the type of mutation in a particular sample. Here, modified vectors with isolated Tg, isolated L and tandem LTg were replicated in HEK 293T cells, single TLS polymerase knockout cell lines (pol **η**, pol **κ**, pol **ι**, pol **ζ**). The TLS efficiency and total mutation profile was obtained, and their respective frequencies were calculated.

4.3 RESULTS

I. ISOLATED THYMINE GLYCOL (Tg)

4.3.1 Complete mutation profile of isolated lesion Tg, when replicated in HEK 293T cells and TLS polymerase knockout cells:

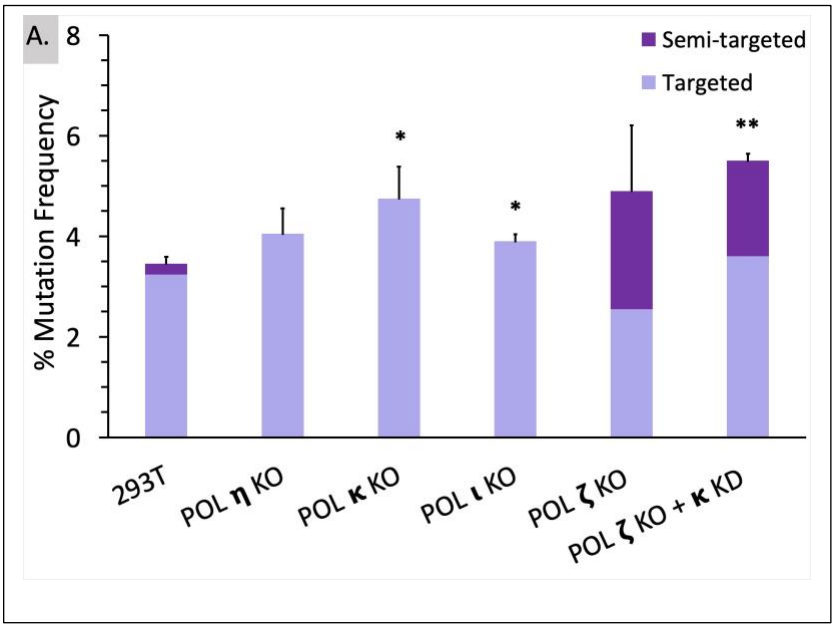


FIGURE 4.2: Total mutation profile of isolated-Tg lesion, when replicated in HEK 293T cells and polymerase knockdown (KD)/knockout (KO) cells. Data obtained from two/three independent experiments. *(p < 0.05); **(p < 0.005)

Figure 4.2 represents the total mutation profile obtained by analyzing the sequencing data. The mutations obtained were categorized into targeted and semi-targeted. Targeted represents the base substitutions/deletions opposite lesion bearing site (Tg). Semi-targeted represents base substitutions/deletions on the 5' and 3' flanking regions of targeted site (Tg). In 293T cells, about

3.4 ± 0.1 % of plasmid progeny recovered were identified as mutants, which include 3.2 ± 0.3 % targeted and 0.2 ± 0.01 % semi-targeted mutations. It was observed that the overall mutation frequencies increased, when Tg was replicated in all single knockout (KO) cell lines (pol **η**, pol **κ**, pol **ι**, pol **ζ**). Among these, greater increase in mutation frequencies, to 4.9 ± 1.3 % and 4.8 ± 0.6 % were found in pol **ζ** KO and pol **κ** KO cells respectively. It was obvious that the targeted mutations were more prevalent that semi-targeted mutations, except for pol **ζ** KO where the they occur in same proportion. Based on the above observations, it was evident that pol **ζ** and pol **κ** possesses an important role in the Tg bypass. Hence, to confirm this observation, Tg was further replicated in a specific double knockout/knockdown (KO/KD) cell line, with pol **ζ** KO + pol **κ** KD. We observed that, the mutation frequency was further increased when pol **ζ** and **κ** were removed, which is 5.5 ± 0.1 %. This data supports the role of pol **ζ** and pol **κ** in the replicative bypass of Tg. It is notable that semi-targeted mutations were observed in 293T, Pol **ζ** KO and pol **ζ**/**κ** KO cells.

4.3.2 Targeted point mutations at isolated-Tg lesion, replicated in HEK 293T cells and TLS polymerase knockout cells:

Figure 4.3 shows the different targeted point mutations of Tg when replicated in various types of cell lines. The common types of base substitutions observed in all the experiments were Tg->A and Tg->G. Other types include, Tg->C and targeted Tg deletion which were found in 293T, pol **ζ** and pol **ζ**/**κ** KO cells. In WT 293T cells, the occurrence of Tg->A was 0.8 ± 0.1 %, which was increased to 3.9 ± 0.1 % in pol **ι** KO and reduced to 0.3 ± 0.03 % in pol **ζ** KO cells. Removal of pol **ζ**/**κ** didn't affect the occurrence of Tg->A but lowered the Tg deletions from 1.6 ± 0.98 % in

WT 293T to 0.65 ± 0.07 %. Significantly, pol ι plays a role in lowering the proportion of Tg->A mutation in WT 293T cells because elimination of pol ι caused this mutation to increase ~ 4-fold.

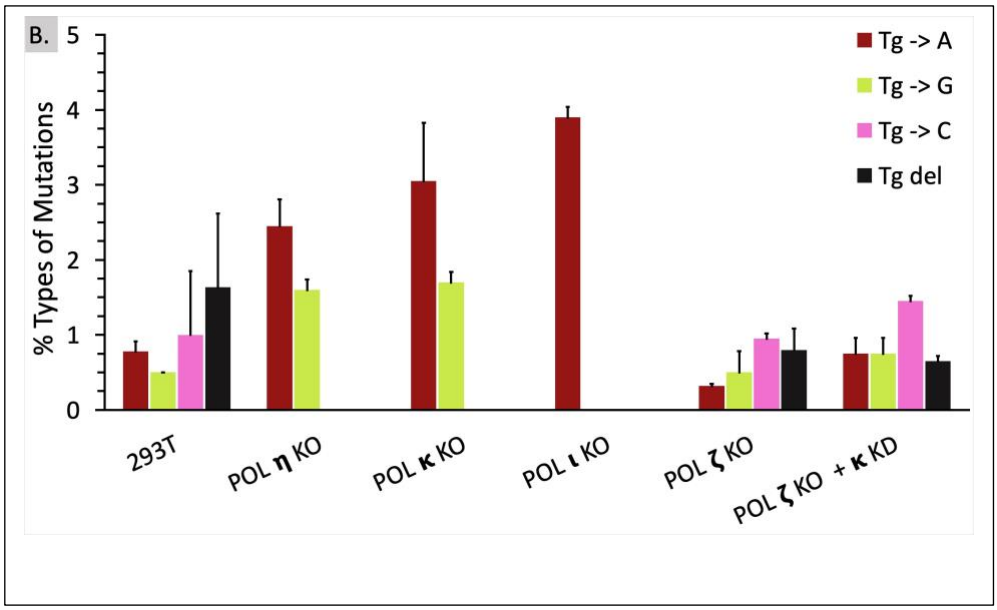


FIGURE 4.3: ***Targeted point mutations at isolated-Tg site, when replicated in HEK 293T and TLS polymerase knockout (KO)/knockdown (KD) cells. Data obtained from two/three independent experiments. * ($p < 0.05$); ** ($p < 0.005$)***

4.3.3 Role of TLS polymerases in the bypass of Tg:

Overall, the mutation frequency is very low ~3.4 % in 293T cells, which means that >95 % of the progeny was bypassed in an error-free manner by the action of replicative polymerases. It can also be inferred that, TLS polymerases ζ and κ add up to promote the error-free bypass of Tg. From these observations, we can propose that pol κ inserts the base opposite Tg and pol ζ in the extension step of TLS mechanism.

II. ISOLATED 2-DEOXYRIBONOLACTONE (L)

4.3.4 Complete base incorporation profile opposite isolated-L lesion, when replicated in HEK 293T cells and TLS polymerase knockout cells:

As described earlier, mutagenicity and cytotoxicity of L and LTg were determined. Since 2-deoxyribonolactone is an oxidized abasic site, the sequencing data obtained, specifying the types of base insertions/deletions opposite L, were categorized as 'full length products' (FLPs) and 'deletions' ((-1)frameshift/ (-2)frameshift mutations). Full length products include, the nucleobases (dA, dG, dT, dC) incorporated opposite targeted site, L.

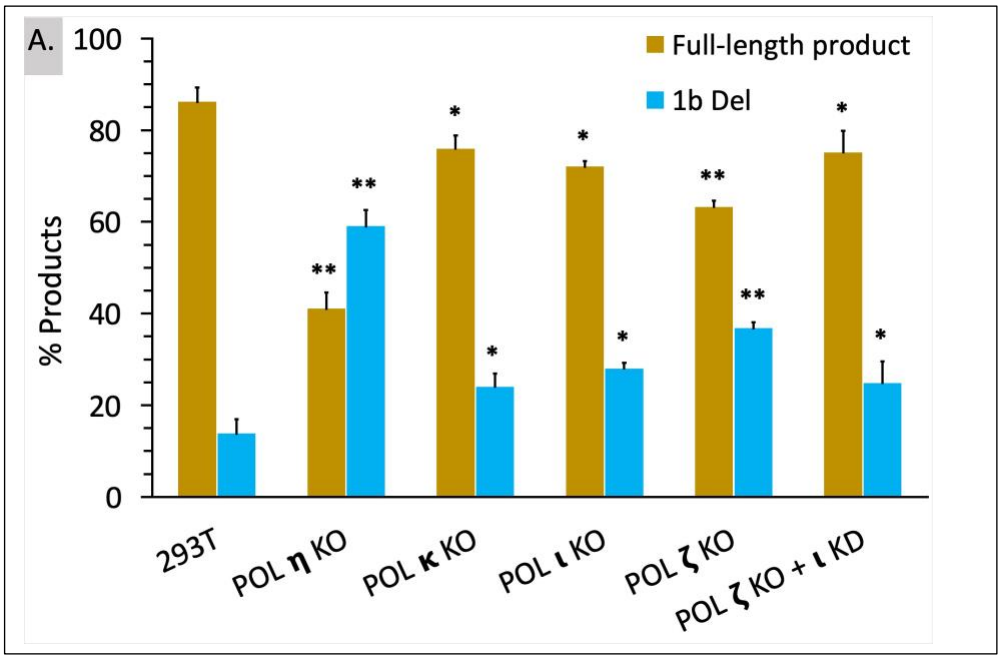


FIGURE 4.4: Percentage progeny recovered including base incorporation (full length products) opposite isolated-L and targeted deletions, when replicated in HEK 293T and polymerase knockout (KO)/ knockdown (KD) cells. Data obtained from two/three independent experiments. * ($p < 0.05$); ** ($p < 0.005$)

Figure 4.4 shows the total mutation profile of isolated 2-deoxyribonolactone with 5' dT flanking base (5'-TL). Among the replicated progeny in 293T cells, nearly 86 ± 3.2 % were FLPs and 14 ± 3.2 % deletions. The percentage of FLPs altered in the TLS polymerase KO cells, reveals that bypass of L occurs by TLS mechanism. Significant reduction in the FLPs to 41 ± 3.5 % was observed in pol **η** KO cells and 63 ± 1.3 % in pol **ζ** KO cells. To compensate the FLPs, the deletions were increased in the inverse order among KO experiments, which was 59 ± 3.5 % in pol **η** KO cells, 37 ± 1.3 % in pol **ζ** KO cells. Notable change in FLPs and deletions was not observed in pol **ι** and pol **κ** deficient cells. Therefore, pol **η** and pol **ζ** possesses important roles in the replicative bypass of isolated L.

4.3.5 Targeted base incorporation opposite isolated-L, in HEK 293T cells and TLS polymerase knockout cells:

Figure 4.5 shows the specific base incorporations (dA, dG, dT, dC) opposite L, included as FLPs, in the 5'-TL sequence. It was observed that, replication of L in 293T cells and polymerase-KO cells, yielded majority of dA incorporation opposite L, which aligns with the 'A-rule' in contrast to in *E.coli* as reported in literature (24). In 293T cells, percentage of dA incorporation was 59 ± 13 %, which was increased in all the pol-KO cells, ranging from 75%-78 %. In spite of the predominant 'A-rule', it is notable that, significant dT incorporation opposite L was found as 35 ± 14 % in 293T cells, which was decreased to 3 %-11 % in KO cells. This shows that the TLS polymerases also promote the incorporation of dT at the expense of dA opposite L in WT 293T cells. Smaller proportions of dG and dC were also incorporated by TLS.

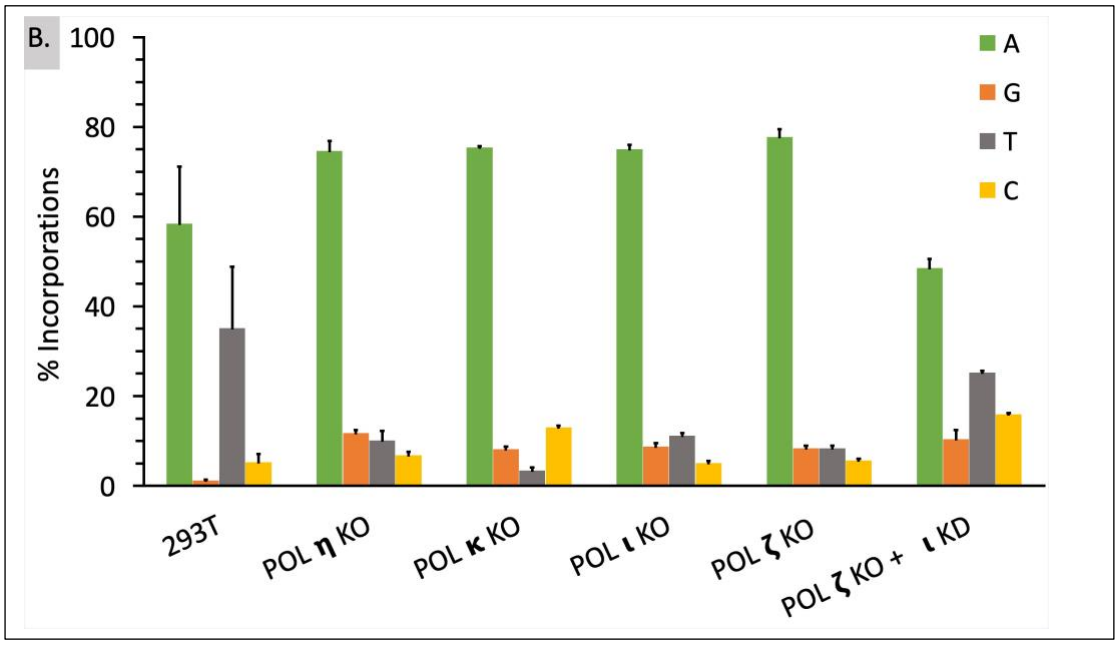


FIGURE 4.5: Targeted base incorporation opposite isolated-L, when replicated in HEK 293T and polymerase knockout (KO)/knockdown (KD) cells. Data obtained from two/three independent experiments. * ($p < 0.05$); ** ($p < 0.005$)

4.3.6 Role of TLS polymerases in the bypass of 5'-TL:

From the FLPs and TLS efficiency data in pol-deficient and 293T cells, it was evident that bypass of L require TLS polymerases. Especially, pol **η** is required in the identification and insertion of nucleobase opposite L to yield FLP, thus reducing the (-1) frameshift mutations. Additionally, pol **η** also improves the survival of the cells. Although pol **ζ** doesn't have a role in the TLS efficiency it is important in the generation of FLPs as shown in figure 4.12. Hence, pol **η** can solely insert and extend the sequence in TLS bypass of L.

4.3.7 Sequence-dependent bypass of L (5'-TL and 5'-CL):

To investigate the role of 5' flanking bases in the bypass of L, additionally a modified vector with 5'-CL adduct was constructed and replicated in 293T cells. The data containing FLPs, deletions and targeted base incorporations were obtained from sequencing analysis. The proportion of FLPs was decreased to 81 ± 0.6 % in 5'-CL from 86 ± 3.2 % in 5'-TL bypass as shown in figure 4.6. The proportion ratio of targeted base incorporations (dA, dG, dT, dC) opposite L remained the same, predominantly with dA (65 ± 1.3 %) and dT (19 ± 2.5 %) in 5'-CL bypass represented in figure 4.7. Therefore, it was evident that the A-rule was still followed even with different 5' nucleobase sequence containing L. Hence, it can be deduced that 5'-CL will be replicated with the similar efficiency by TLS polymerases.

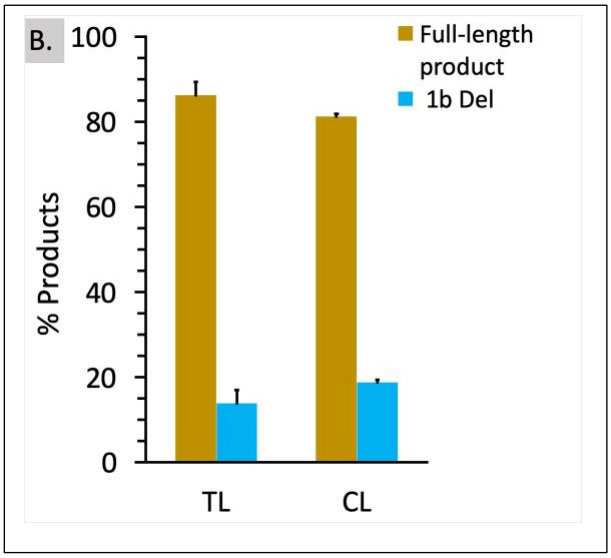


FIGURE 4.6: Percentage progeny recovered corresponding to 5'-TL and 5'-CL, including base incorporations (full length product) opposite isolated-L and deletions, when replicated in HEK 293T cells. Data obtained from two/ three independent experiments.

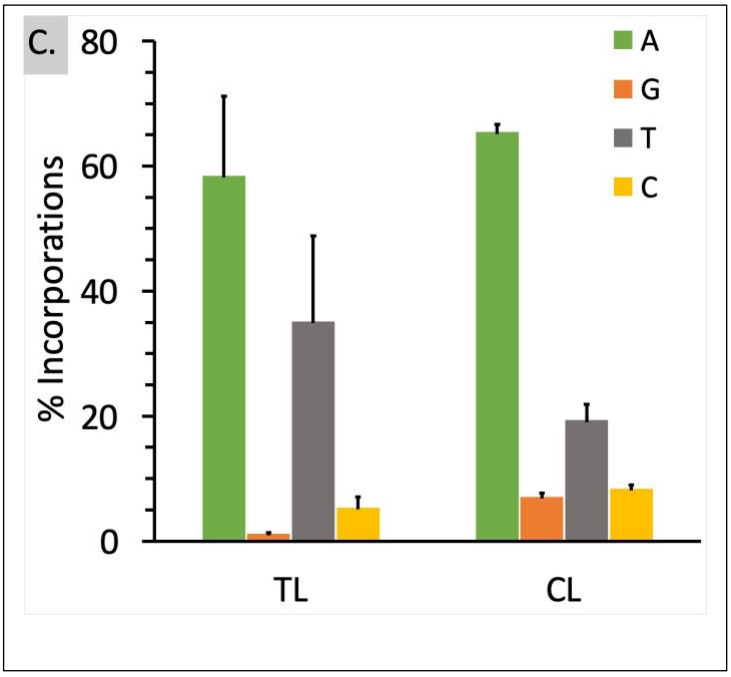


***FIGURE 4.7**: Targeted base incorporation opposite L, when 5'-TL and 5'-CL were replicated in HEK 293T cells. Data obtained from two/three independent experiments.*

III. TANDEM LESION: 5'-LTg-3'

4.3.8 Complete base incorporation profile of tandem lesion, 5'-LTg-3', when replicated in HEK 293T cells and TLS polymerase knockout cells:

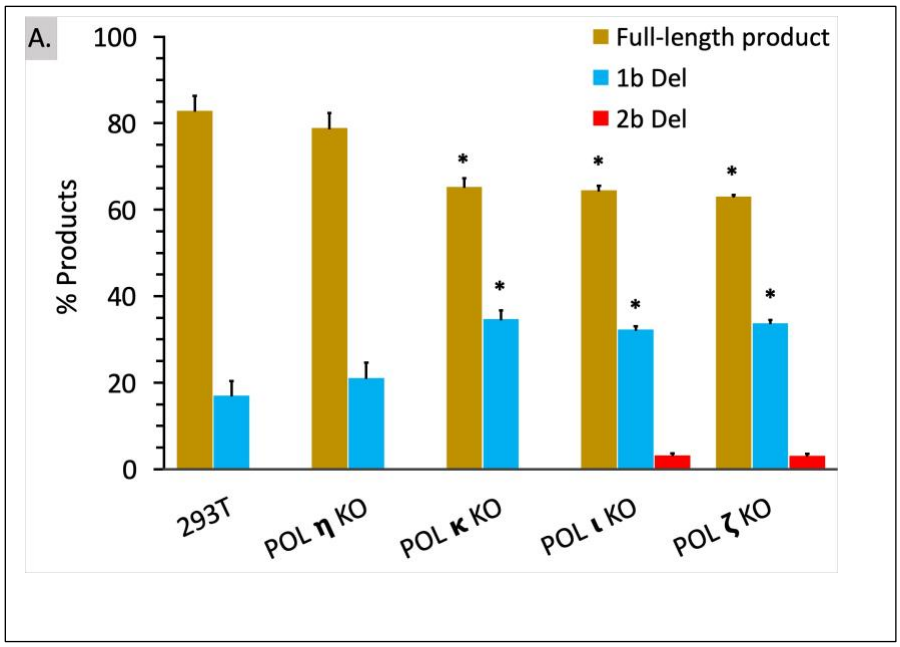


FIGURE 4.8: Percentage of progeny recovered corresponding to tandem lesion 5'-LTg-3', including base incorporation (full length product) opposite 'L' and deletions (1b and 2b), when replicated in HEK 293T and polymerase knockout cells. Data obtained from two/three independent experiments. * ($p < 0.05$); ** ($p < 0.005$)

Complete mutation profile of LTg was categorized into full length products (FLPs), L-deletion (1b deletion), L & Tg-deletion (2b deletions), as represented in the figure 4.8. In 293T cells, the fraction of FLPs was 83 ± 3.4 %, reduced to 63 ± 0.3 % in pol **ζ** deficient cells, to 65 % in both pol **κ** and pol **ι** KO cells. Elimination of Pol **η** had no effect on the proportion FLPs. The most notable observation was the incidence of 2b-deletions (L & Tg) in only pol **ζ** and pol **ι** KO cells, which was 3.2/3.3 ± 0.4 % respectively. Also, the percentage of 1b-deletions (L), increases from

17 ± 3.3 % in 293T cells to 34 ± 0.7 % and 32 ± 0.7 % in pol **ζ** and pol **ι** KO cells respectively. Hence, it can be deduced that pol **ζ** and pol **ι** play a critical role in the elimination of the frameshift mutations in the tandem lesion bypass.

4.3.9 Base incorporation opposite 5'-Targeted (L) component as part of tandem lesion, in HEK 293T cells and TLS polymerase knockout cells:

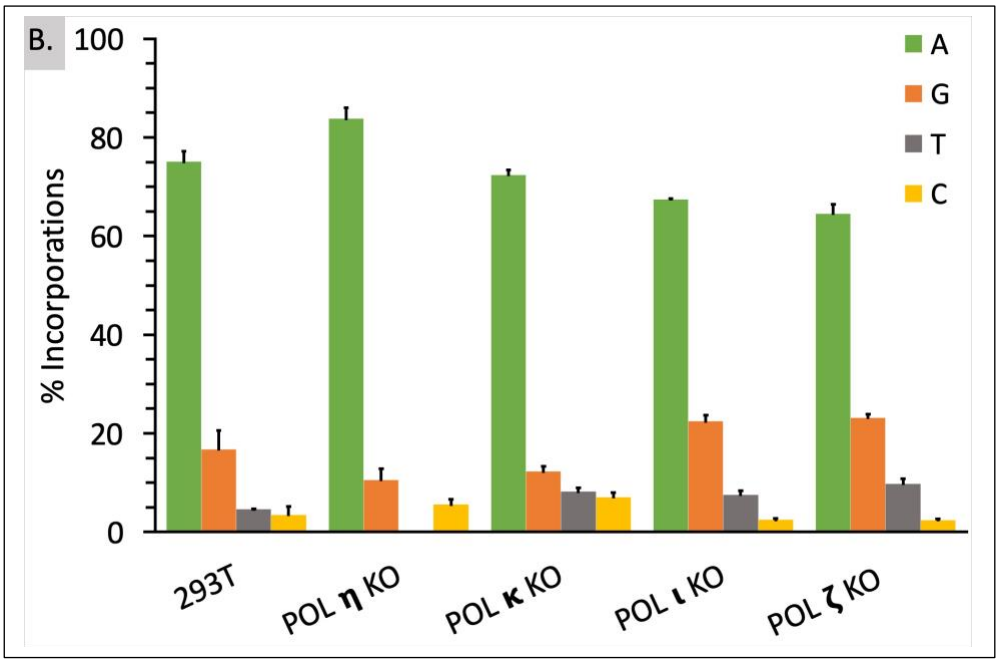


***FIGURE 4.9**: Base incorporation opposite 5'-targeted (L) site as part of LTg, when replicated in HEK 293T and polymerase knockout cells. Data obtained from two/three independent experiments. * ($p < 0.05$); ** ($p < 0.005$)*

From the figure 4.9, dA incorporation opposite 5'-targeted (L) within LTg, in 293T cells was 75 ± 2.1 %. This was reduced to 65 ± 1.9 % in pol **ζ** KO, 68 ± 0.1 % in pol **ι** KO and increased to 84 ± 2.3 % in pol **η** KO cells. Therefore, dA incorporation opposite L was predominant in the bypass

of tandem lesion, followed by dG and to a small extent of dT and dC. Thus, A-rule is facilitated by pol ζ and pol ι opposite 5'-targeted (L).

4.3.10 Mutation profile of 3'-Targeted (Tg) component as part of tandem lesion in HEK 293T cells and TLS polymerase knockout cells:

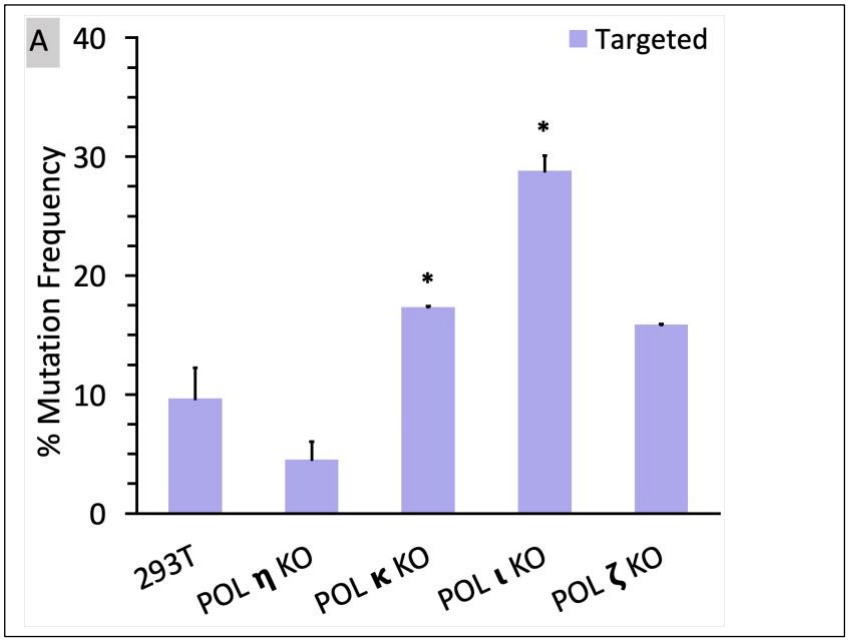


FIGURE 4.10: Mutation profile of 3'- Targeted (Tg) component as part of LTg, when replicated in HEK 293T and polymerase knockout (KO) cells. Data obtained from two/three independent experiments. * ($p < 0.05$); ** ($p < 0.005$)

Figure 4.10 represents the total mutation frequencies of 3'-targeted (Tg) as part of LTg replication. The data shows that the predominant incorporation is dA, which was >87 % in 293T cells. Elimination of pol κ, pol ζ and pol ι resulted in the increased mutation frequencies to 17 ± 0.1 %, 16 ± 0.1 %, 29 ± 1.3 % respectively from 10 ± 2.6 % in 293T cells. It was reduced to 4.5 ± 1.5 %

in pol **η** KO cells. Despite the different trends, pol **ι** was evidently responsible for the error-free bypass of Tg within the tandem lesion, followed by pol **κ** and pol **ζ**.

Figure 4.11 represents the 3'-targeted Tg plot with point mutations and deletions obtained from different experiments. Notably, Tg deletions were observed only in pol **ζ** and pol **ι** KO cells at similar frequencies. Point mutations, Tg->A, Tg->G and Tg->C were found in all the experiments and at varied frequencies. However, elimination of pol **ι** resulted in the increase of mutation frequencies, Tg->A and Tg->G to 16 ± 0.1 % and 13 ± 1.3 % respectively. From these observations, it was evident that in case of 3'-targeted (Tg), pol **ζ** and pol **ι** promote the error-free bypass in 293T cells.

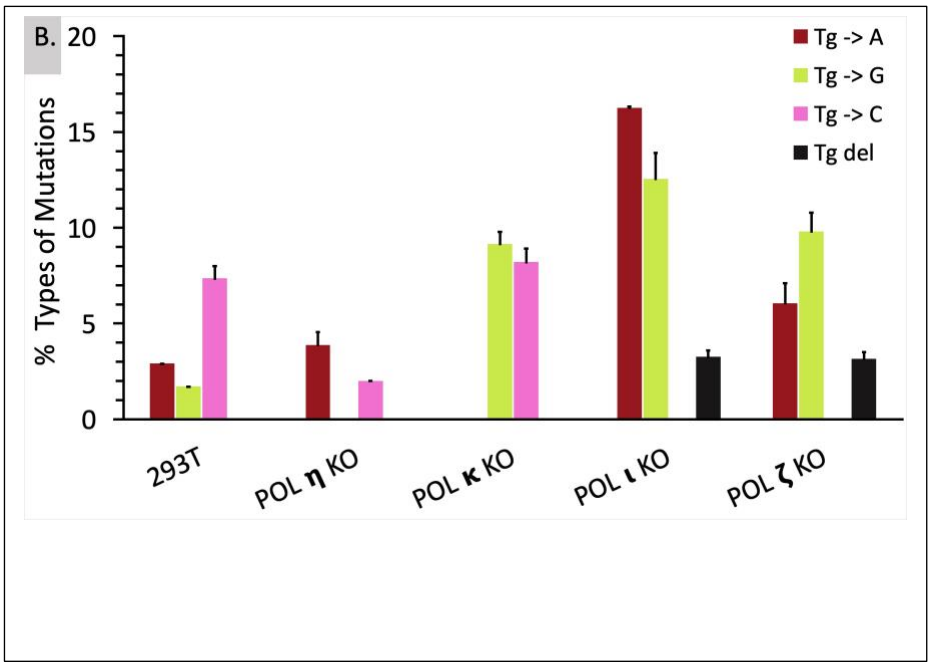


***FIGURE 4.11**: 3' Targeted (Tg) point mutations when 5'-LTg-3' was replicated in HEK 293T and TLS polymerase knockout (KO) cells. Data obtained from two/three independent experiments. * ($p < 0.05$); ** ($p < 0.005$)*

4.3.11 Role of TLS polymerases in the bypass of tandem lesion, LTg:

From the different representations of the data corresponding to the components 5'-L and 3'-Tg within the tandem lesion, it can be deduced that LTg replication in 293T cells occur through TLS mechanism and that it is a strong replication block. In this study, it was evident that pol **ζ** and pol **ι** take over the responsibility of efficient bypass of the components, 5'-L & 3'-Tg in tandem, that is by incorporation dA opposite both the lesion sites. This was demonstrated by the corresponding single knockout experiments, which increased the (-2) frameshift mutations in 5'-L and mutation frequencies in 3'-Tg.

4.3.12 TLS efficiency of Tg, 5'-TL and LTg:

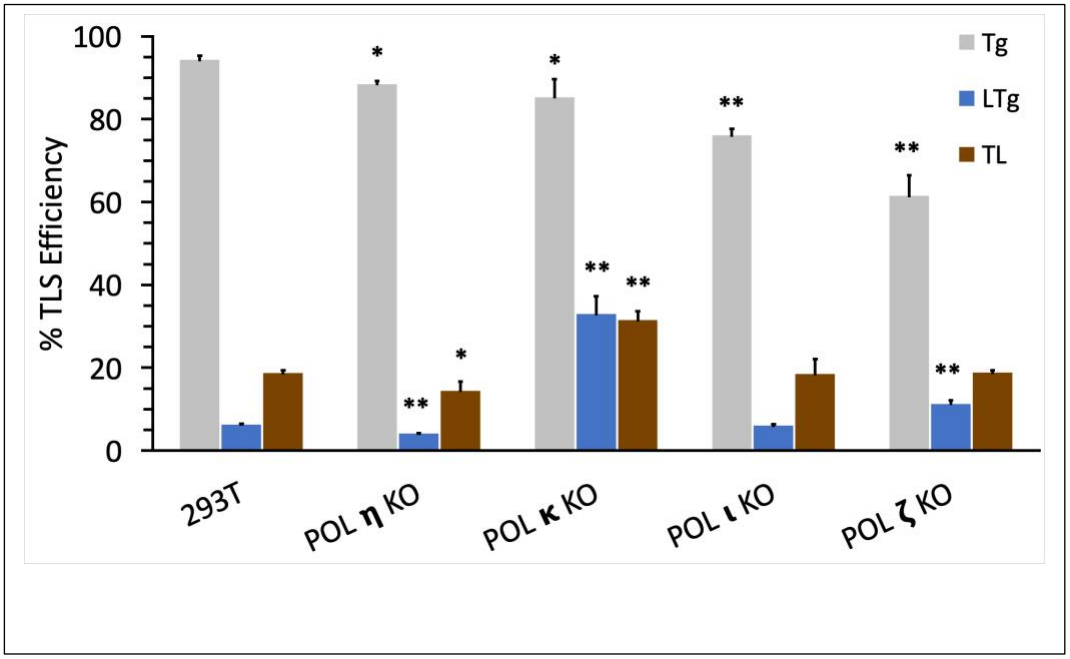


FIGURE 4.12: TLS efficiencies of Tg, LTg and L in HEK 293T and polymerase knockout (KO) cells. Data obtained from two/three independent experiments. * ($p < 0.05$); ** ($p < 0.005$)

Figure 4.12 shows the TLS efficiencies of the three adducts, isolated Tg, tandem lesion 5'-LTg-3' and isolated lesion 5'-TL in the respective order. Firstly, TLS efficiency in the bypass of Tg was discussed, followed by L and then 5'-LTg-3'.

TLS efficiency of Tg was reduced in pol-KO cells compared to ~95 % in 293T cells, which shows that Tg is not a strong replication block. Removal of Pol **ζ** resulted in maximum reduction in TLS efficiency ~40 %, followed by 20 % in Pol **ι** KO, 10 % in pol **κ** KO and 6 % in pol **η** KO cells. Hence, it was evident that pol **ζ** has an important role in the bypass of Tg with higher survivability. Secondly, the TLS bypass efficiencies of L in the form of two sequence dependent adducts, 5'-TL and 5'-CL was discussed. TLS efficiency of 5'-TL in 293T cells was 19 ± 0.6 %, which was reduced to 15 ± 2.1% in pol **η** KO cells. TLS efficiency was not affected when pol **ι** and pol **ζ** was eliminated. However, it was increased to 32 ± 2.1 % in pol **κ**-deficient cells. Based on these variations in TLS efficiencies of polymerase deficient cells, it can be assumed that, pol **η** possess an important role in the replicative bypass of L and that recruitment of pol **κ** blocks the replication. In comparison, 5'-CL replication reduced the cell survival ~4-fold in 293T cells, i.e., 5.5 ± 0.3 %. It can be hypothesized that 5'-flanking sequence distorted the DNA in a way that the TLS efficiency was compromised, hence reducing the survivability.

Lastly, TLS efficiency of tandem lesion, LTg in 293T cells was 6 ± 0.2 %, it drastically increased ~5-fold in pol **κ** deficient cells. There exist fluctuations in the TLS efficiencies in other pol-deficient cells, ranging from 4-12 %. Hence, similar to isolated L, recruitment of pol **κ** might result in the formation of unproductive replication complexes which block the replication of tandem lesion (LTg).

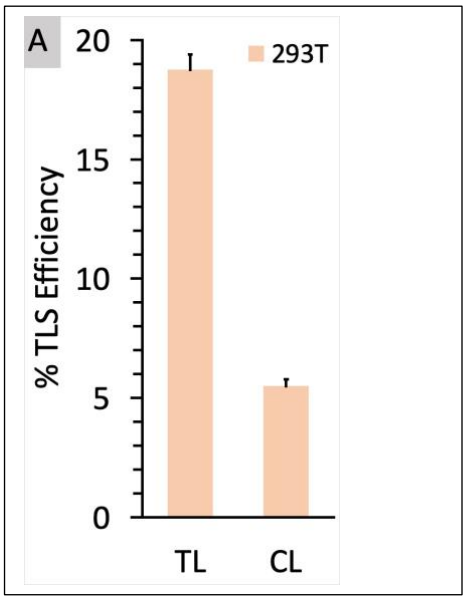


***FIGURE 4.13**: TLS efficiencies of 5'-TL and 5'-CL in HEK 293T cells. Data obtained from two/three independent experiments.*

4.4 DISCUSSION:

Oxidized abasic lesions form an important class of DNA damages, when left unrepaired can be bypassed by TLS mechanism. In this study, we investigated the TLS bypass of one such tandem lesion with two components, 5'-L and 3'-Tg, along with isolated lesions L and Tg in HEK 293T cells. We have generated three single stranded plasmids with site specific incorporation of isolated L, isolated Tg, tandem LTg adducts and replicated in HEK 293T cells. Earlier studies revealed that isolated lesion Tg was a strong replication block and also weakly mutagenic (< 1 %) in *E.coli (10,13)*. It was also reported that the mutagenicity of Tg increases in *E.coli* when it is a part of tandem lesion (5). Earlier studies on the TLS bypass of isolated Tg located on both leading and lagging strands of the DNA by Prakash and colleagues (13) in human cells revealed that, pol **κ** and pol **ζ** act together to bypass Tg, that the former inserts the correct nucleotide opposite Tg (non-mutagenic) and the latter extends in an incorrect manner beyond the lesion (mutagenic) (13). In this research on the TLS bypass in human cells, the isolated Tg was found mutagenic and it increased when it was a part of tandem lesion as 3'-Tg. It also aligns with the previous study, that the TLS polymerases necessary for the isolated Tg bypass were pol **κ** and pol **ζ**, as proved by the increased mutagenicity of Tg in double knockout pol **κ**/**ζ** experiment. Further, the TLS bypass of LTg in HEK 293T cells and the mutagenicity of 3'-Tg and 5'-L were analyzed together and separately, which showed that pol **ζ** and pol **ι** was responsible for efficient bypass. The higher percentage of dG incorporated opposite 3'-Tg in WT 293T cells can be explained due to the error-prone nature pol **ι**. Further, pol **ζ** and pol **ι** play an important role in the elimination of (-2) frameshift mutations (deletion of L & Tg) in the replication of tandem lesion. Hence, the isolated Tg and tandem LTg containing adducts were efficiently replicated by different TLS polymerases,

with pol **ζ** being the common one and it can be speculated that it participates in the extension step of TLS.

Previous studies on isolated 2-deoxyribonolactone (L) showed that it was bypassed in *E.coli (19,24,50), S. cerevisiae (21)* and human cells (51). In *E.coli*, the survival of the cells with L, depends on competitive bypass by SOS pol V, result in deletions and dG/dA substitutions. On the other hand, Pol II/ pol IV, play a role in the replication past the lesion and also in the elimination of - 1 frameshift mutations (deletions) (24,50). The incidence of deletions was particularly observed with respect to the local sequence 5' to L, that is 5'-TL and 5'-CL. Deletions were only observed in 5'-CL (24). In this study, we have explored the similar effect of the local sequence, 5'-TL and 5'-CL in HEK 293T cells, where deletions were observed in both adducts. However, targeted deletions increased in 5'-CL compared to 5'-TL adduct. The overall proportion of FLP to the targeted deletions remained the same when both the adducts were replicated in 293T cells. Moreover, the TLS efficiency differed significantly in 5'-CL which dropped to ~27 % compared to 5'-TL. The dominant base incorporations were dA, followed by dT for both sequence types. The base incorporations opposite L were not influenced by the 5'-local nucleobase which was similar to *E.coli,* as reported in (50). Taking this into consideration, the TLS bypass and the corresponding base incorporations was studied for a single model of 2-deoxyribonolactone, 5'-TL in 293T cells.

In 5'-TL adduct, the major polymerases which were found to play a role in the TLS were pol **η** and pol **ζ**. This was evident from the pol **η** KO and pol **ζ** KO experiments, where the occurrence of (-1) frameshift mutations increased by 4-fold and 2.5-fold respectively. It was also noted that all the TLS polymerases promote the incorporation of dA, following 'A-rule', along with significant dT incorporation opposite L. Similarly, TLS mechanism followed 'A-rule' with respect to 5'-L component within tandem lesion, but dT incorporation was very less. *In vitro* studies of isolated L

and tandem LTg bypass using X-family DNA polymerases revealed that, pol **β** alone can bypass isolated L by dA incorporation and that it was strongly blocked by the tandem lesion, LTg. It was also speculated that B-family polymerase, pol **ζ** aid in its extension (51). Another tandem lesion bypass with different components, 5'-8oxodG, Tg-3' was reported in *E.coli*, which reported that the overall mutagenicity was increased and TLS efficiency was reduced by half (52). To our best knowledge, there was no study reported on the role of B and Y-family polymerases and its efficiency, in the bypass of oxidized abasic lesion, in tandem with thymine glycol, 5'-LTg-3' in HEK 293T cells, which we report here for the first time using site specific mutagenesis approach. We observed that the TLS efficiency of tandem lesion was dropped ~3-fold compared to isolated L and ~15-fold to isolated Tg in WT 293T cells. It was also noted that (-2) frameshift mutations were eliminated by either pol **ι** or pol **ζ**. The major polymerase responsible for the bypass is extension by pol **ζ**, in all the three lesions investigated here. Pol **ζ** incorporates dA opposite abasic site and was necessary in the bypass similar to *E.coli,* as reported in (53). Significantly, pol **ι** was necessary in the error-free bypass of 3'-Tg within tandem lesion. It is also worth mentioning the unique role of pol **κ** in the bypass of isolated L and LTg, where its absence, increased the TLS efficiency. Possibly, pol **κ** generates unproductive replicative complexes which may blok the replication. Pol **η** also has an important role in the tandem bypass which is an analogue of SOS pol V in *E.coli*. These results indicate that TLS polymerases are essential in the bypass of tandem lesions formed in human cells and are extremely cytotoxic.

4.5 CONCLUSION:

Oxidized abasic sites formed by the hydroxyl radicals are considered as important class of DNA damages. They can be efficiently repaired by long-patch BER and NER. If left unrepaired, the lesions were replicated by TLS. Tandem lesions, a part of clustered lesions is mutagenic as well as cytotoxic in humans. In this study, we explored the TLS bypass of isolated Tg, isolated L and tandem LTg. Here, we summarize our findings as isolated Tg was not a strong block of replication in human cells and was weakly mutagenic. But the mutagenicity of Tg increased when it is part of tandem lesion. TLS bypass of Tg was achieved by pol **κ** and pol **ζ** (54). Isolated L was found to be a stronger block of the replication and that pol **η** & **ζ** were necessary. Further, the tandem lesion, LTg which was reported to occur more frequently in the cells, was a very strong block of DNA replication. The smaller proportion of recovered progeny from the tandem lesion bypass, was aided by pol **ι** and pol **ζ** for both components 5'-L & 3'-Tg. The roles of TLS polymerase altered when the adducts were replicated singularly vs in tandem. Hence, tandem lesions formed as a result of oxidation or ionizing radiation has a radical effect in the DNA replication fidelity and cell survival.

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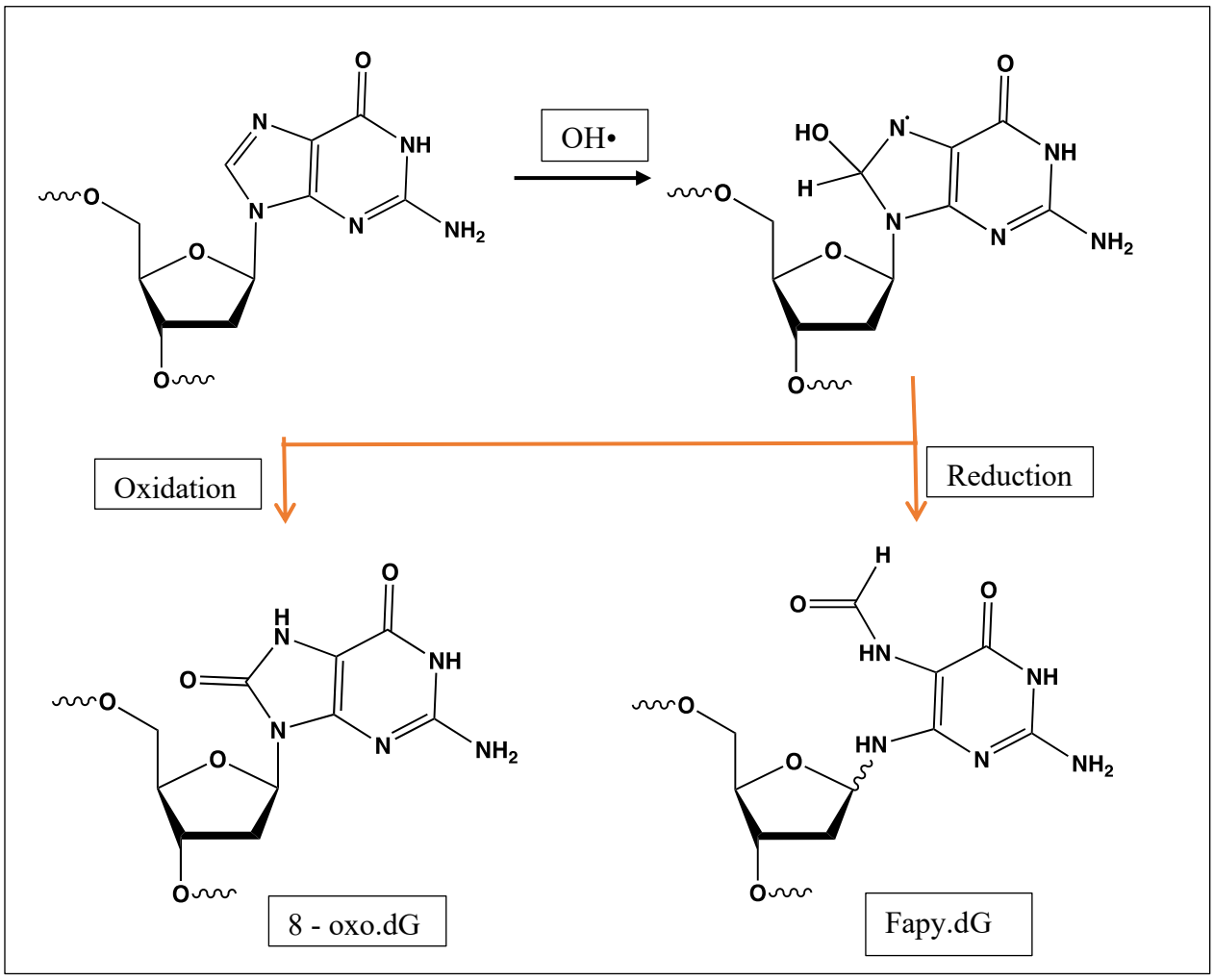
CHAPTER-5

Translesion synthesis bypass of Fapy.dG lesion (*N*-2, 6-diamino-4-hydroxy-5-formamidopyrimidine) in human cells

5.1 Introduction:

Oxidative stress in the cells generates a plethora of DNA lesions from the modified purines/pyrimidines and backbone sugar moieties (1-4). Various lesions include formation of DNA protein cross-links, tandem lesions, abasic sites and strand breaks (4). If left unrepaired, these damages can result in oxidative induced damage is caused by the exposure of DNA to oxidizing agents, which generates ROS. One such common highly reactive ROS is hydroxyl (OH•) radical (1, 2, 5). OH• add to the double bonds of purines at C8 position to generate N7-radicals, which upon further reactions produce 8-oxopurines and formamidopyrimidines (Fapy) (6-9). Of these, dG is more susceptible to oxidation because of their lower reduction potential (10). Therefore, formation of 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxo.dG), N-(2-deoxy-D- pentofuranosyl)-N-2, 6-diamino-4-hydroxy-5-formamidopyrimidine) (Fapy.dG) are more prevalent. The relative proportions of 8-oxo.dG to Fapy.dG depends on the oxidative conditions at the time of lesion formation (11, 12). The common N7-radical intermediate undergo one-electron oxidation to form 8-oxo-dG and other direction is to undergo **β**-fragmentation followed by reduction to form Fapy.dG (2, 13, 14). For example, when DNA is exposed to **γ**-radiolysis, greater proportion of 8-oxo.dG is formed under aerobic conditions and Fapy.dG under anoxic conditions (7, 13). Whereas, exposure of human leukemia cells to ionizing radiation forms Fapy.dG, ~3-fold higher than 8-oxo.dG (13, 15, 16). Both are observed in comparable amounts, when DNA is irradiated with UV light (17).



SCHEME 5.1: Formation of 8-oxo.dG and Fapy.dG from oxidation of deoxyguanine by a hydroxyl radical. The N7 radical intermediate formed undergo either one electron oxidation to form 8-oxo.dG or one electron reduction to form Fapy.dG

Extensive studies on the mutagenesis of 8-oxo.dG were done previously and Fapy.dG to some extent in *E.coli (15)*,mammalian (18) and human (19) cells. SOS induction in *E.coli* increases the bypass efficiency of 8-oxo.dG, but has no effect in the Fapy.dG (15). Moreover, Fapy.dG is found to be weakly mutagenic in *E.coli (15)* in contrast to its bypass in mammalian COS-7 cells (18). It was also reported that Fapy.dG is ~25 % more mutagenic than 8-oxo.dG in COS-7 cells (18). A

study on the mutagenicity of both dA and dG derived adducts of 8-oxopurines and formamidopyrimidines was conducted by Kalam and colleagues (18), where it was reported that dA-derived adducts are weakly mutagenic than its corresponding dG adducts. It was also reported that, Fapy.dG is a potent mutagenic lesion and 8-oxo.dA is a weak one in COS-7 cells. *In vitro* TLS studies proved that 8-oxo.dG is bypassed in an error-free manner by X-family polymerase **λ** with correct dC incorporation opposite the damaged base. It was rather proved in HEK 293T cells, that error-free bypass of 8-oxo.dG (20, 21) and error-prone bypass of Fapy.dG was facilitated by pol **λ** in different sequence contexts (19). Hence, Pol **λ** is also responsible for the induction of G–>T transversions in the replication of Fapy.dG. However, the influence of B and Y family TLS polymerases in HEK 293T cells is not yet explored, which is primary focus of this study.

Fapy.dG adduct exists as both *syn*- and *anti*- conformations around the glycosidic bond which influences the complementary base pair interaction. It also exists in **α**- and **β**- anomeric forms, which are interconvertible and influence the cytotoxicity (22-26). Earlier thermodynamic studies (23) on the anomeric influence in the replication of Fapy.dG showed that, **α** anomer destabilizes the double helix and thus strongly blocks the replication *in vitro*. This is assumed to be because of the position of the lesion outside the helix and hence is cytotoxic. However, **β** anomer also blocks the DNA replication *in vitro* to a lesser extent and is found to predominant in DNA (13, 22, 23). Studies performed in human cells revealed that the mutagenicity is clearly obtained from the predominant **β**- anomeric form of Fapy.dG adduct (13). On the other hand, it is also influenced by the *syn*- and *anti*-conformations adapted by the adduct (27). It was reported that 8-oxo.dG exists in the energy-driven *syn*- conformation which leads to G->T mutations and *anti*- in the non-mutagenic bypass. In contrast to this, the energy-favorable conformation of Fapy.dG is *anti*- which incorporates dC opposite to it (4, 15, 27, 28). Fapy.dG also exists in the *syn*- conformation which

is responsible for the occurrence of G->T mutations (4, 15, 27). Overall, Fapy.dG is considered to be highly mutagenic in COS-7 cells (18, 26).

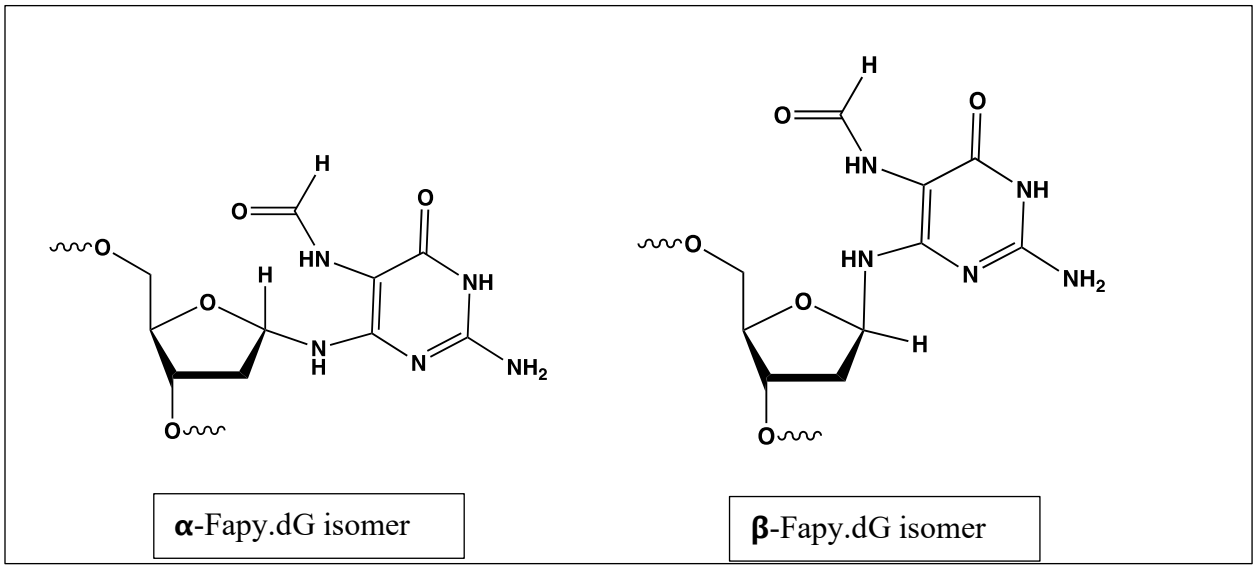


FIGURE 5.1: The two anomeric forms-α and β of Fapy.dG adduct in the DNA

5.2 Repair:

Formamidopyrimidines and 8-oxopurines are efficiently repaired by BER mechanism as reviewed in (4) and (13), where various DNA glycosylases participates in the excision of the damaged bases (29-31). In *E.coli*, three DNA glycosylases participate in the repair of oxidative DNA lesions, namely, endonuclease III (*Nth*), endonuclease VII (*Nei*) and formamidopyrimidine DNA glycosylase (*fpg*), whose analogues found in humans as hNTH1, hNEIL and hOgg1 respectively (30, 32). *Fpg* efficiently excise 8-oxodG, Fapy.dG and Fapy.dA at similar kinetic rates (8) but with different recognition criteria (28) was reported *in vitro* by Widerholt and colleagues (33). But hOgg1 can repair only 8-oxo.dG and Fapy.dG, not Fapy.dA (34, 35). It was reported that both the enzymes efficiently repairs the Fapy.dG:dC substrate than Fapy.dG:dA mispair and that the latter was repaired by MutY/MutYH enzyme, a part of enzyme cluster called 'GO system' similar to the 8-oxo.dG (36). MutY/MutYH enzyme removes the complementary nucleotide dA from the mispair, hence is unable to repair the FapydA:dA substrate (33, 37). It was reported that the latter was repaired by glycosylases Nei, (*E.coli*) (38) & NEIL (humans) (39). hNEIL family includes three enzymes (NEIL1, NEIL2, NEIL3) specific for different lesions (32). In this case, NEIL1 is involved in the repair of wide variety of oxidative purines/pyrimidines including Fapy.dA because of its broader specificity (40). hOgg1 specifically repair the adducts on the double strand DNA and hNEIL1 acts on the single stranded DNA forks or bubble structures that form during DNA replication and transcription (39, 41). In the DNA with multiple lesions, enzymes Nth and Nei can efficiently excise only Fapy.dA in *E.coli (38)*. Whereas in humans, hNEIL1 alone excise both Fapy.dG and Fapy.dA adducts (42), however, hNTH1 has no role (43).

5.3 Fapy.dG adduct:

Fapy.dG was prepared using a novel synthetic approach by Greenberg and colleagues *(unpublished)* and was site specifically incorporated in a 12-mer oligonucleotide. The 12-mer employed in this study represents a part of *p53* gene sequence and the lesion site was located at a 273 codon (CG*T). The resulting adduct was analyzed by mass spectrometry. The structure and the sequence were shown below.

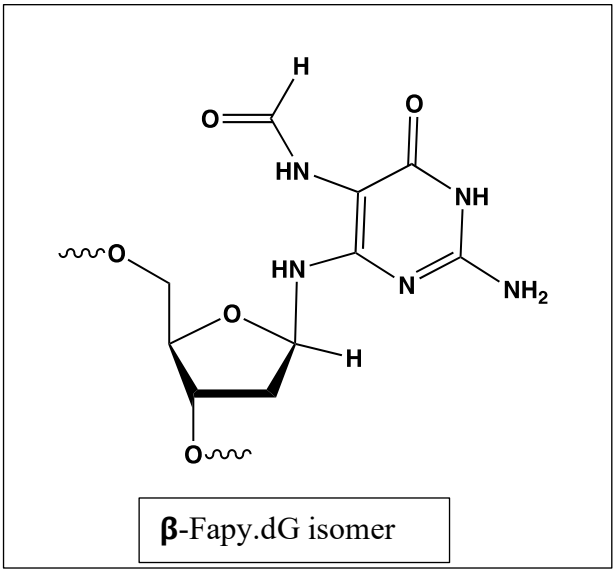


FIGURE 5.2: Structure of β-anomer of Fapy.dG lesion

5.4 RESULTS:

5.4.1 Replication of Fapy.dG in human cells:

Single stranded pMS2 vector with inserted 12-mer adduct containing Fapy.dG was constructed and quantified using agarose gel electrophoresis. This vector was replicated in HEK 293T cells along with TLS-polymerase knockout/knockdown cells and were collected. The DNA was harvested, replicated in *E.coli* DH10B cells and the mutants were screened by Southern blot hybridization. These selected progeny were commercially sequenced to determine the type and the position of the mutation in the adduct. The sequence of the Fapy.dG is shown below,

5' – GTGCG*TGTTTGT - 3'
(G* - Fapy-dG)

5.4.2 TLS efficiency:

Figure 5.3 shows the TLS efficiencies of Fapy.dG in HEK 293T cells and various single polymerase knockout (KO) cells (pol **η**, pol **ι**, pol **κ**, pol **ζ**). The TLS efficiency in 293T cells was 72 ± 1.4 %, was reduced to 29 ± 3.5 % in pol **κ** KO cells, 47 ± 4.9 % in pol **η** KO cells and 53 ± 0.7 % in pol **ζ** KO cells. TLS efficiency of Fapy.dG was not affected when pol **ι** was eliminated. From these values, it was evident that pol **κ** play an important role in the bypass of Fapy.dG and promote survival of the cells.

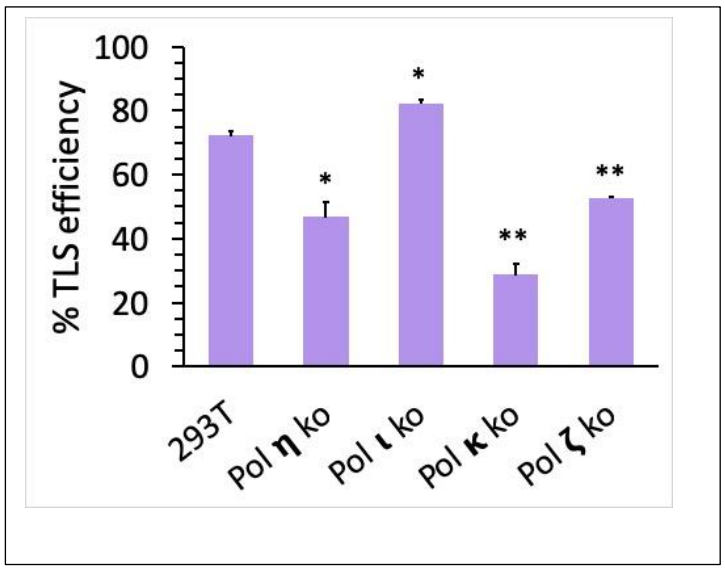


FIGURE 5.3: TLS efficiency of Fapy.dG in HEK 293T cells along with four single polymerase knockout cells. Results obtained from three individual experiments.

5.4.3 Complete mutation profile of Fapy.dG in HEK 293T and polymerase-deficient cells:

The mutational data obtained after the sequencing was categorized into targeted and off-targeted mutations as shown in the figure 5.4. Targeted mutations represent the point base mutations/deletions at the lesion site and off-targeted mutations represent the base substitutions/deletions on the 5' and 3' flanking regions of the adduct. In 293T cells, mutation frequencies (MF) correspond to targeted mutations was 10.3 ± 0.4 %, reduced to 3.6 % in pol **ζ** KO cells, 4.8 ± 0.2 % in pol **ι** KO cells and 6.4 ± 0.3 % in pol **κ** KO cells. Off-targeted mutations in HEK 293T cells was 0.6 ± 0.4 %, increased to 1.1 ± 0.1 % in pol **ι** KO cells. No change in the MF was observed in pol **ζ** KO cells and they were eliminated in pol **κ** KO cells. From these observations, it can be deduced that pol **ζ**, pol **ι** and pol **κ** may play a critical role in the error-prone bypass of Fapy.dG in HEK 293T cells.

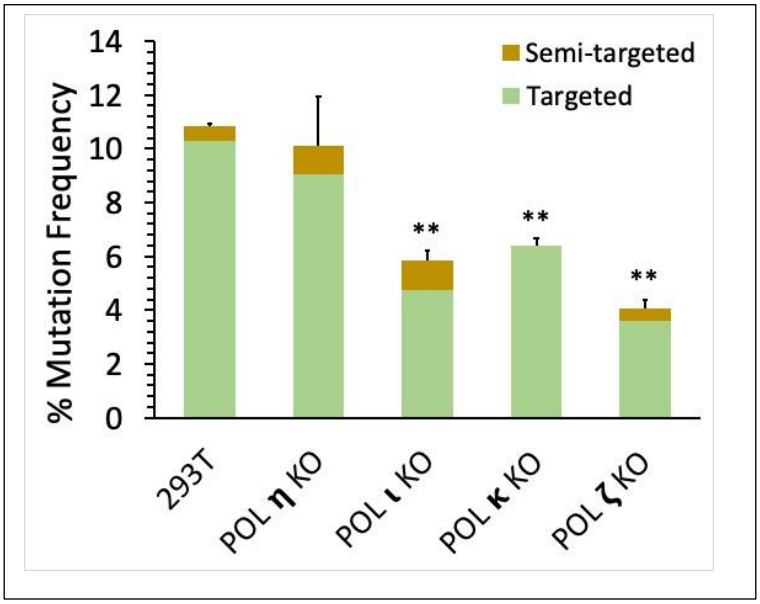


FIGURE 5.4: ***Mutation frequencies correspond to targeted and off-targeted mutations, when Fapy.dG was replicated in HEK 293T cells and single polymerase-knockout cells. Data represent three independent experiments.***

5.4.4 Mutation frequencies of targeted point mutations at Fapy.dG lesion site, when replicated in HEK 293T and polymerase-deficient cells:

Among the targeted mutations, base substitutions were prevalent than deletions as represented in the figure. Of the targeted base substitutions, G->T transversions possesses greater MF in 293T cells, that is 7.4 ± 0.1 % of G -> T, 1.5 ± 0.1 % of G -> C, 0.9 ± 0.1 % G -> A. The incidence of significant proportion of all these targeted substitutions were observed in pol **ζ**, pol **ι** and pol **κ** cells. Proportion of G->T transversions was 2 % in pol **ζ** KO cells, 3.1 ± 0.1 % in pol **κ** KO cells and 3.3 % in pol **ι** KO cells. These were found to be relative to other base substitutions in these cells. From the data (figure 5.5), it can be deduced that pol **ζ**, pol **ι** and pol **κ** play a role in the error-prone bypass and can be assumed that Fapy.dG adapts *syn*- conformation when it was bypassed by TLS polymerases which explains the predominant incorporation of dA opposite lesion.

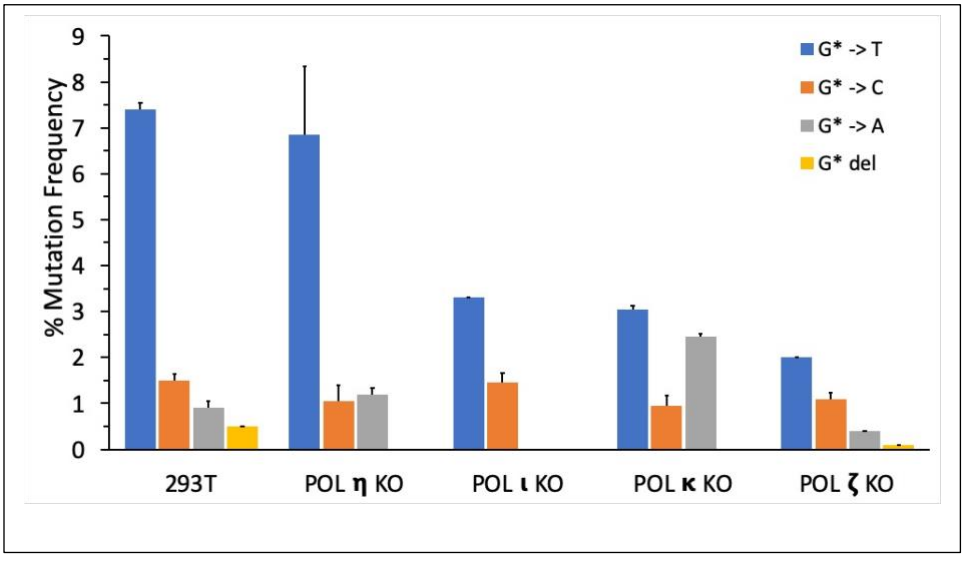


FIGURE 5.5: Targeted point mutations at Fapy.dG site, when replicated in HEK 293T and polymerase-deficient cells. Data obtained from three independent experiments.

5.5 SUMMARY:

From the data obtained from WT 293T and single polymerase-knockout (pol **η**, pol **ι**, pol **κ**, pol **ζ**) experiments, it can be hypothesized that pol **ι**, pol **κ**, pol **ζ** act synergistically in the bypass of Fapy.dG in an error-prone manner. It is also notable that pol **η** didn't affect the bypass in a significant way. In addition to this, Fapy.dG tend to adapt both *syn*- and *anti*- confirmations in human cells. As reported in the literature (26, 28), the energy-favorable *anti*-conformation of Fapy.dG is still predominant which explains >85 % of replication to proceed in an error-free manner.

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Appendix

List of Publications

Naldiga, S., Ji, S., Thomforde, J., Nicolae, C. M., Lee, M., Zhang, Z., Moldovan, G-L., Tretyakova, N. Y., Basu, A.K. (2019) Error-prone replication of a 5-formylcytosine-mediated DNA-peptide cross-link in human cells. *J. biol.chem.*, **294** *(27)*, 10619-10627.

Naldiga, S., Huang, H., Greenberg, M. M., Basu, A. K. (2019) Mutagenic effects of a 2-deoxyribonolactone-thymine glycol tandem DNA lesion in human cells. *Biochemistry*, 10.1021/acs.biochem.9b01058.

Ji, S., Fu, I., **Naldiga, S.**, Shao, H., Basu, A. K., Broyde, S., Tretyakova, N. Y. (2018). 5-formylcytosine mediated DNA-protein cross-links block DNA replication and include mutations in human cells.